Feruloylated polysaccharides in the primary cell walls of *Festuca arundinacea*

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Abbreviations

A	Compound A
AIR	Alcohol insoluble residue
Ara	Arabinose
Ara_f	Arabinofuranose
AraH	Arabinitol
AX's	Arabinoxylans
B	Compound B
BAB	PC solvent; butanone/acetic acid/boric $acid_{(sat.\ in\ water)}$ (9 : 1 : 1, v/v/v)
BAW	PC solvent; butan-1-ol/acetic acid/water (12 : 3 : 5, v/v/v)
BEW	PC solvent; butan-1-ol/ethanol/water (20 : 5 : 11, v/v/v)
BPW	PC solvent; butan-1-ol/pyridine/water (4 : 3 : 4, v/v/v)
B	Compound B: feruloylated disaccharide
B_S	Compound B_S: de-feruloylated disaccharide
B_S-ol	Compound B_S after reduction
Chlorbutanol	1,1,1-Trichloro-2-methylpropan-2-ol
Compd	Compound
Compd D'	Monoesterified compound D
"Compound D"	Putative compound D
cpm	Counts per minute
DMSO	Dimethyl sulfoxide
DP	Degree of polymerisation (number of sugar residues per molecule)
dpm	Disintegrations per minute
EAW	PC solvent; ethyl acetate/acidic acid/water (10 : 5 : 6, v/v/v)
EPW_1	PC solvent; ethyl acetate/pyridine/water (8 : 2 : 1, v/v/v)
EPW_2	PC solvent; ethyl acetate/pyridine/water (10 : 4 : 3, v/v/v)
EryH	Erythritol
EtOH	Ethanol
FAXX	Fer-Ara-Xyl_2; Xyl_2: from xylan backbone; Fer-(Xyl)-Ara; (Xyl): from side chain
Fig(s).	Figure(s)
Fer	Feruloyl residue
FT	Fourier Transformation (with IR spectroscopy)
Gal-1,3-AraH	β-D-Galactosyl-(1→3)-D-arabinitol
GC	Gas chromatography
Glc	Glucose
GPC	Gel permeation chromatography
HPLC	High pressure liquid chromatography
IR	Infrared spectroscopy
Isoprimeverose	α-D-xylopyranosyl-(1→6)-D-glucose
kBq	Kilobecquerel (disintegrations/μs)
α/β-Me-Xyl	α/β-Methyl-xyloside

m_{Ara}	Mobility relative to arabinose (in PE systems)
Maltose	α-D-Glucopyranosyl-(1→4)-D-glucose
Maltotriose	α-D-Glucopyranosyl-(1→4)-D-α-glucopyranosyl-(1→4)-D-glucose
MBq	Megabecquerel (disintegrations/μs)
M_r	Molecular weight
MS	Mass spectrometry
$NaBH_4$	Sodium borohydride
$NaIO_3$	Sodium iodate
$NaIO_4$	Sodium periodate
nd	not determined
NMR	Nuclear magnetic resonance (spectroscopy)
p-NP	*p*-Nitrophenol
p-NP α/β-Xyl	*p*-Nitrophenyl-α/β-D-xyloside
NTS	Non-Triton scintillation cocktail
PAHBAH	*p*-Hydroxybenzoic acid hydrazide
PAXX	*p*-Cou-Ara-$(Xyl)_2$
PC	Paper chromatography
p-Cou	*p*-Coumaroyl residue
PCW	Plant cell wall
PE	Paper electrophoresis
POPOP	1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO	2,5-Diphenyloxazole
R_{Ara}	Mobility relative to arabinose (in PC systems)
2-d-Rib	2-deoxyribose
2-d-RibH	2-deoxyribitol (= 2-deoxyarabinitol)
RIC	Relative ion current
R_f	Mobility relative to solvent front (in PC systems)
RPC	Disposable reverse-phase chromatography column
rpm	Revolutions per minute
SCFA's	Short chain fatty acids
Sigma	Sigma Chemical Co.
TFA	Trifluoroacetic acid
ThrH	Threitol
TLC	Thin layer chromatography in benzene/acetic acid (9:1)
TS	Triton scintillation cocktail
XG2	Isoprimeverose
Xyl	Xylose
Xyl_2	Xylobiose
XylH	Xylitol
Xylobiose	β-D-Xylosyl-(1→4)-D-xylose
Xyl_p	Xylopyranose
ν	Frequence
§	Section
Φ	Phenol
1_s	De-feruloylated compound 1

CONTENTS

Abstract

Hydroxycinnamic acids are present in land plants as soluble and as insoluble (cell wall bound) esters. The most common hydroxycinnamic acids found in cell walls are ferulic and *p*-coumaric acids. These compounds are present particularly in Gramineae, where they are linked to arabinoxylan. The roles of cell wall bound ferulic acid have not been completely elucidated.

Recently it has been reported that feruloylated arabinoxylans of cultured *Festuca* cells are relatively resistant to the enzyme mixture Driselase whereas Driselase can act successfully on other graminaceous cell walls (e.g. maize and barley) to release feruloylated oligosaccharides (e.g. ~79% was solubilised from maize) such as Fer-Ara-Xyl (FAX) and Fer-Ara-Xyl_2 (FAXX). The resistance of *Festuca* feruloyl-arabinoxylans to Driselase means that only a very low percentage of the feruloyl groups were released as FAX and FAXX. The feruloylated material was solubilised by Driselase (~80% as well) but in the form of relatively high molecular weight conjugates. The question of why *Festuca* feruloyl-arabinoxylan cannot be digested by Driselase to fragments such as FAX and FAXX to any great extent has now been investigated.

The major feruloylated oligosaccharide (compound B) released from *Festuca arundinacea* cell walls by mild acid hydrolysis showed the unusual structure 5-*O*-(*E*)-feruloyl-[*O*-β-D-xylopyranosyl-(1→2)]-L-arabinose. The (1→2)-linkage was established by $NaIO_4$-oxidation and by PE of the $NaBH_4$-reduced compound in molybdate-buffer alongside marker sugars with (1→2)- and (1→3)-linkages; the (*E*)-feruloyl ester group by its characteristic fluorescence and UV absorption; the 5-*O*-linkage by partial hydrolysis of compound B to the known compound, 5-*O*-feruloyl-L-arabinose (compound A); and the β-linkage by hydrolysis with β-xylosidase. The structure was confirmed by NMR spectroscopy and methylation analysis.

Other products released from *Festuca* cell walls by mild acid included compound A itself and several other feruloyl esters (C to H) larger than compound B. Compounds C, D and E each gave compound B upon treatment with Driselase; their chromatographic properties suggested that C, D and E had additional sugar residues attached to a core of compound B. Graded saponification indicated that D and E (but not C) also possessed a non-feruloyl ester group, probably acetate.

The biosynthesis of feruloylated arabinoxylans was studied by feeding [^{3}H]arabinose and [^{14}C]ferulate to suspension cultures of fescue and by assaying the radiolabelled polymer-bound Ara, Fer-Ara (=A) and Fer-(Xyl-)Ara (=B) groups over a time-course. The Fer-Ara and Fer-(Xyl)-Ara groups became ^{3}H-labelled with a lag of <5 min, confirming that feruloylation is intraprotoplasmic. The Fer-Ara and Fer-(Xyl-)Ara groups both became ^{14}C-labelled with a lag of <1 min, suggesting that, to produce Fer-(Xyl-)Ara, the Ara is first xylosylated and then feruloylated.

Since compound B has an unusual structure, the question then pursued was whether compound B is unique to *Festuca arundinacea* or it is more widespread. All monocots tested (21 graminaceous and 1 member of the Palmae) showed the presence of compound B.

To investigate whether compound B is likely to confer resistance to microbial degradation of *Festuca* arabinoxylan or not, [*pentosyl*-^{3}H]B and [*feruloyl*-^{14}C]B were incubated *in vitro* with rat caecal contents. Degradation products (Fer-Ara, Ara-Xyl, xylose, arabinose and ferulic acid) could be monitored at a very early stage, showing that all enzymes necessary for a rapid breakdown of compound B are present in the gut micro-organisms.

It had been suggested that there was a link between the unusual structure of compound B, the feruloylated arabinoxylan of fescue and the relative Driselase "resistance" and therefor would not be expected to confer enzyme resistance on the polymer. However, compound B itself is not resistant to digestion by gut micro-organisms.

1. Introduction

1.1 What is a plant cell wall?

The cell is the basic unit of life. The main difference between a plant and an animal cell is its cell wall - or is it not? Helen Stafford (1991) asked in "The Plant Cell" whether "its boundary starts with the outer cell wall or with the plasmalemma". Is the cell wall therefore intra- or intercellular? Opinion is divided on this. Stafford (1991) instigated the debate with her letter concerning a discussion of how to define a plant cell. Staehelin (1991) responded to this by stating that "a cell is a small, self-propagating compartment that is bounded by a semipermeable membrane and is filled with cytoplasm, a concentrated aqueous solution of chemicals" doing so by consulting reference books such as "Molecular Biology of the Cell", (Alberts *et al.*,1989) and the Encyclopedia Britannica (1989). His further argument is that "we kill the cell by removal of the plasmalemma but removal of its cell wall does not". Therefore it is clear (to him and other authors: e.g. Newcomb, 1980) that "the plant cell wall is a special form of extracellular matrix". Sack (1991) continued the discussion by referring to plant cell authorities such as Katherine Esau and Ray Evert, who define the cell as a compartment consisting of protoplast and cell wall, essentially implying that the cell wall is intracellular.

In "The Last Word" on the plant cell discussion (to date) Robinson (1991) also argues for an intracellular definition of a cell wall: "Certainly enzymatic removal of the cell wall does not kill a plant cell, but, as a protoplast, the cell cannot divide and regenerate without producing a new wall. This new wall is initially located intracellularly in the form of the centrifugally growing cell plate, and it first becomes an extracellular entity when it fuses with the plasma membrane of the maternal cell wall. Plant life includes division and growth, and the cell wall plays an integral role in these fundamental processes". The author of this thesis supports the idea that the plant cell wall is part of the plant cell.

It has been suggested (Bolwell, 1988; Roberts, 1989) that the term cell wall should be replaced by extracellular matrix with dynamic properties. Nevertheless, "cell wall" still predominates, most likely for convenience, and will be used in this thesis.

1.2 General aspects of cell walls

Plant cell walls can be seen to be analogous to the extracellular matrix of animal cells (Sack, 1991). They have long been perceived as being a passive and inert outer layer. This view must be challenged in view of the distinctly active role the cell wall exhibits in determining cell shape and maintaining rigidity and size of the cell. It can be argued that cell walls control the growth rate of cells and consequently the growth rate of the whole plant. How does growth take place? In order to answer this interesting question it is of great importance to strengthen our knowledge of the structure and biosynthesis of cell walls.

"We believe that only when the biochemistry of primary cell wall structure and synthesis is understood, will a fundamental understanding of plant growth be possible" (Darvill *et al.*, 1980).

The cell wall in general is composed of a series of layers starting from the cell plate (after cell division), which turns into the middle lamella containing material from both daughter cells. The middle lamella is less than 30 nm thick (Brett & Waldron, 1990). More and more material will be laid down to form the primary cell wall (between 0.1 and 1.0 μm thickness, Brett & Waldron, 1990) as long as the cells are growing. The primary wall controls the rate of growth. Special types of cells in which the primary wall is the only wall (Wilson, 1993) are e.g. parenchyma cells (with unthickened primary cell walls) and collenchyma cells (thickened primary walls, but no lignification). Parenchyma cells are found in many tissues e.g. apical meristematic cells of roots and shoots. Collenchyma cells strengthen young plant organs; they show an unusually elongated shape (Alberts *et al.*, 1986). Primary cell walls are typically made up of approximately 90% polysaccharides and 10% protein (McNeil *et al.*,1984).

Once cells have reached a certain size, a third layer may be deposited: the secondary cell wall. Cells with secondary walls undergo differentiation into particular cell types with special functions. Such functions are maintenance of mechanical strength and cell shape and the control of intercellular transport. Secondary cell walls themselves can function as food reserves. In seeds, cotyledon cell walls may contain xyloglucan or α-(1→3)-arabinan or β-(1→4)-linked mannan, whose products the seedlings need during and after germination. Furthermore, cells, especially cells with secondary walls respond to pathogen attack and environment stress (Brett &

Waldron, 1990; Bolwell, 1993). Phytopathogens, for instance, attack plants by enzymic digestion of their cell walls. The plant responds by making its wall indigestible by laying down lignin, callose, silica, extensin, etc. (Bell, 1981). The lignification (for biosynthesis see § 1.3.2.4.1) starts in the middle lamella and expands into the primary and secondary cell wall. Typically cells with highly lignified walls are sclerenchyma and xylem. Sclereids are a type of sclerenchyma cells which make up some seed coats or the endocarp of stone fruits which gives the flesh of pears its gritty texture (Lawrence, 1992).

1.3 Cell wall polysaccharides

The polysaccharides of plant cell walls are distinguished by their great heterogeneity. They contain at least nine types of monosaccharides (Aspinall, 1980; Darvill *et al.,* 1980; Waldron & Brett, 1985, Table 1.1) held together by different glycosidic bonds.

The names of the polysaccharides refer to the quantitatively dominant glycosyl residues (Darvill *et al.,* 1980). Not all of these polymers are present in the same quantity in plant cell walls. In addition they are connected to other wall components such as proteins, phenolics, water, etc. to form a complex network. In the following sections some of these cell wall components are examined in more detail.

Once extracted from the wall, most of the polymers are water-soluble (Fry, 1986) with the exception of cellulose. Consequently there must be covalent and/or non-covalent interpolymer cross-linkages. It is known that such cross-links are responsible for cell adherence (Blaschek & Franz, 1983) and indigestibility (Fry, 1984).

Non-covalent linkages can be formed by H-bonding and/or ionic bonds, in particular Ca-bridges. Ionic bonds in general are possible because of the strong electrostatic force of attraction between groups with negative and positive charge (Holleman & Wiberg, 1985). The major negatively charged groups occurring in wall polysaccharides are the carboxyl groups of glucuronic acid in xylans and galacturonic acid in pectins (Fry, 1989).

Bond	Polysaccharides in which the bond is found
Ara α-(1→2)-Ara	Arabinan
Ara α-(1→3)-Gal	Arabinogalactan I
Ara α-(1→3)-Xyl	Arabinoxylan
Fuc α-(1→2)-Gal	Xyloglucan
Gal β-(1→5)-Ara	Arabinoxylan
Gal β-(1→4)-Xyl	Xylan
GalA α-(1→4)-GalA	Homogalacturonan
GalA α-(1→2)-Rha	Rhamnogalacturonan I
Glc β-(1→4)-Glc	Cellulose, Glucomannan, Xyloglucan
Glc β-(1→3)-Glc	Callose
Glc β-(1→4)-Man	Glucomannan, Galactoglucomannan
GlcA β-(1→6)-Gal	Arabinogalactan II
GlcA β-(1→2)-Man	Glucuronomannan
GlcA α-(1→2)-Xyl	Glucuronoxylan
Man β-(1→4)-Glc	Glucomannan, Galactoglucomannan
Man α-(1→4)-GlcA	Glucuronomannan
Man β-(1→4)-Man	Glucomannan, Galactoglucomannan
Rha α-(1→4)-GalA	Rhamnogalacturonan I
Xyl β-(1→2)-Ara	Xylan
Xyl (1→4)-Gal	Xylan
Xyl α-(1→6)-Glc	Xyloglucan
Xyl β-(1→4)-Xyl	Xylan

Table 1.1: Glycosidic bonds found in plant cell wall polysaccharides (Aspinall, 1980; Darvill *et al.,* 1980; Waldron & Brett, 1985).

The formation of H-bonds is possible between hydroxyl, amino and amido groups, either intermolecular (like the glucan chains in a microfibril) or intramolecular. A single H-bond has a binding energy of just 10 - 20 kJ/mol in comparison to 460 kJ/mol for a covalent H-O bond (Lehninger, 1987). The formation of H-bonds is probably non-enzymic (Fry, 1989) whereas a pectinesterase is involved forming Ca-bridges between different galacturonan units (Yamaoka *et al.,* 1983).

Covalent linkages can be formed by oxidative coupling of polymer bound phenolics (Geissmann & Neukom, 1971) e.g. tyrosine and ferulic acid (see below).

As stated earlier (§ 1.2), the cell wall is composed of a series of layers (the middle lamella, the primary wall and finally the secondary wall). The components of these layers can be classified either as crystalline or non-crystalline aggregations.

1.3.1 Cellulose

The crystalline phase is relatively homogeneous and made up of cellulose which is an unbranched β-(1→4)-glucan (Fig. 1.1) and forms the skeletal framework of the cell wall (Fry, 1989):

-β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→4)-β-

Fig. 1.1: Partial structure of cellulose.

Approximately 30-50 individual glucan chains (held together by hydrogen bonding) form a microfibril which excludes water without which the association could be disrupted (Bolwell, 1993). It is not yet clear whether the cellulose molecules are in parallel (all reducing ends facing to one end) or in antiparallel configuration (Brett & Waldron, 1990). The celluloses of primary and secondary walls are quite similar except in degree of polymerisation as the celluloses of monocot and dicot cell walls (Darvill *et al* .,1980).

1.3.2 The matrix

Hemicelluloses, pectins, glycoproteins (e.g. extensin) and phenolics (e.g. *p*-coumaric acid, ferulic acid, lignin) are classified as the non-crystalline phase, which is quite heterogeneous (designated the matrix). The matrix contains 70-80% of the dry weight of the primary wall material (Albersheim, 1978).

In the following sections, the structural aspects are discussed. In addition to their structural role, hemicelluloses and pectins also have a functional role: they are a source of intercellular signalling molecules (Darvill *et al.,* 1980). For example, specific xyloglucan oligosaccharides were found to inhibit cell elongation (Fig. 1.4) when present at nM concentrations (McDougall & Fry, 1989 and 1990).

1.3.2.1 Hemicelluloses

Alkaline treatment of cell walls will release hemicelluloses by cleavage of the hydrogen bonds between hemicellulose and cellulose microfibrils (Brett & Waldron,1990). In contrast to cellulose, most hemicelluloses have short side chains and are acetylated (Wood & McCrae,1986; Fry, 1988). In arabinoxylan, for instance, side chains of α-L-arabinose and α-D-glucuronic acid are present. Acetyl groups are removed during alkaline extraction (Wood & McCrae,1986). It has been suggested that acetylation may decrease the digestibility of cell walls (Bacon *et al.,* 1975). The acetylation has the effect that fewer H-bonds can be formed, so the bonding between cellulose and hemicelluloses is not as strong as within a microfibril. The main hemicelluloses are:

Callose. This is produced in sieve tube elements, and in many other cells in response to wounding. Callose is a β-(1→3)-glucan (Fig. 1.2).

-β-Glc-(1→3)-β-Glc-(1→3)-β-Glc-(1→3)-β-Glc-(1→3)-β-

Fig. 1.2: Partial structure of callose.

Xyloglucans. They are the best-studied wall matrix polysaccharides (Bolwell, 1993). They are the major hemicellulosic polysaccharides of primary cell walls of dicots but they are only minor components in primary cell walls of grasses (Bacic *et al.,*1986), most of the hemicellulose of monocots being mixed-linkage glucans and arabinoxylans (Fry,1985). Xyloglucans have a backbone of β-(1→4)-glucopyranose residues which is highly branched (Fig. 1.3). About ¾ of the glucopyranose residues are substituted at *O*-6 with α-D-xylopyranose residues (York *et al.,*1990).

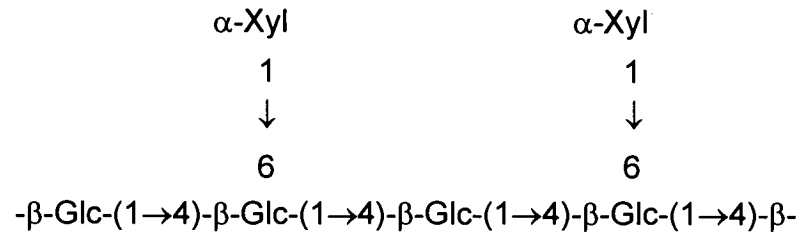


Fig. 1.3: Partial structure of a xyloglucan.

Xyloglucan nonasaccharide (XXFG, Fig. 1.4) acts as an anti-auxin at a concentration of 1 - 10 nM (York *et al.,* 1984; McDougall & Fry, 1989) in that it inhibits cell elongation. It was found that the α-xylose residue of the non-reducing end of the molecule is not essential for the inhibitory activity (Augur *et al.,* 1992).

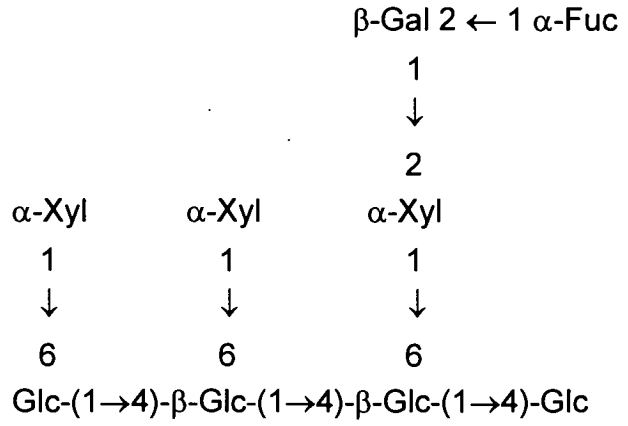


Fig. 1.4: Xyloglucan nonasaccharide (XXFG) as anti-auxin.

Xylan. The backbone of xylans is composed of β-(1→4)-xylose residues (Woolard *et al.,* 1976; Wilkie & Woo, 1977; Das *et al.,* 1981); xylan is the major constituent of the secondary cell walls of dicots (Whistler & Richards, 1970) and dominant in primary cell walls of grasses (Darvill *et al.*, 1980; Brown & Fry, 1992). The xylans from both sources appear similar (Darvill *et al* ., 1980) except that in primary cell walls of dicots arabinose residues are mainly attached to *O*-2 and in grasses to *O*-3 of the xylan backbone (Bacic *et al.,* 1988; Carpita & Gibeaut, 1993; Buchala *et al.*, 1972; Comtat *et al.,* 1974;

Darvill *et al.,* 1980; Amadò & Neukom, 1985; Labavitch & Ray, 1978; Shibuya & Misaki, 1978; Bacic *et al.,* 1986).

To this xylan backbone a wide variety of side chains are attached: single α-L-arabinofuranose residues are attached to position *O*-3 (Andrewartha *et al.,* 1979; Woolard *et al.,*1976; Wood & McCrae, 1986; Gowda & Sarathy, 1987); single α-D-glucopyranoseuronic acid or 4-*O*-methyl-α-D-glucopyranoseuronic acid residues attached to position *O*-2 of the xylose residues (Darvill *et al.*, 1980; Dalessandro & Northcote, 1981) (Fig. 1.5).

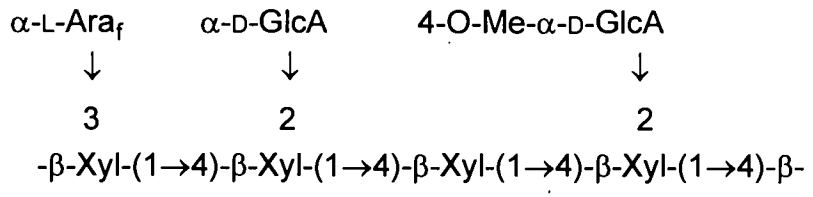


Fig. 1.5: Partial structure of a graminaceous glucuronoarabinoxylan.

These glucuronoarabinoxylans (GAX) are probably the most common hemicellulosic polymers in monocot primary cell walls (Bolwell, 1993; Perlin, 1951; Montgomery & Smith, 1955; Mares & Stone, 1973; Bacic *et al.,* 1986).

The α-L-arabinofuranose residues (predominantly terminally linked: Bacic & Stone, 1981) are partially esterified with ferulic acid (Buchala *et al.,* 1972; Smith & Hartley, 1983; Mueller-Harvey *et al.,* 1986). In the presence of peroxidase and hydrogen peroxide these feruloyl components can form diferuloyl crosslinks between wall polysaccharides (see below).

The backbone of arabinoxylans consists of 40 to 50 β-(1→4)-interlinked D-xylose residues (Buchala *et al.,* 1972; Dalessandro & Northcote, 1981). The α-L-arabinofuranose side chains cause the molecule to be water soluble (Neukom, 1976). Precipitation can be achieved by removal of the arabinose residues by an α-L-arabinofuranosidase or by mild acid hydrolysis (Neukom *et al.,* 1967; Amadò & Neukom, 1985) (Fig. 1.6).

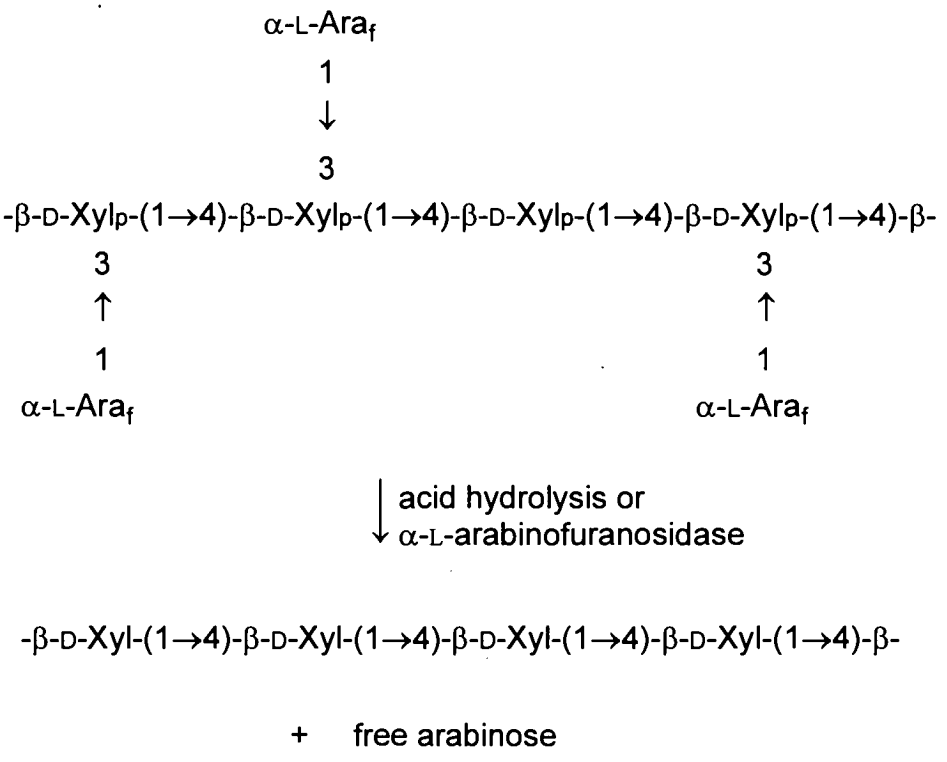


Fig. 1.6: Arabinoxylan treated with mild acid or with α-L-arabinofuranosidase leads to precipitation of the xylan.

1.3.2.2 Pectins

Pectins (pectic polysaccharides) have the ability to form gels (Rees & Welsh, 1977). Apart from galacturonic acid residues (homogalacturonan), which are the dominant components of this group, there are neutral arabinans, galactans, arabinogalactans, and two types of rhamnogalacturonans (Bolwell, 1993). Figure 1.7 shows part of a rhamnogalacturonan I chain.

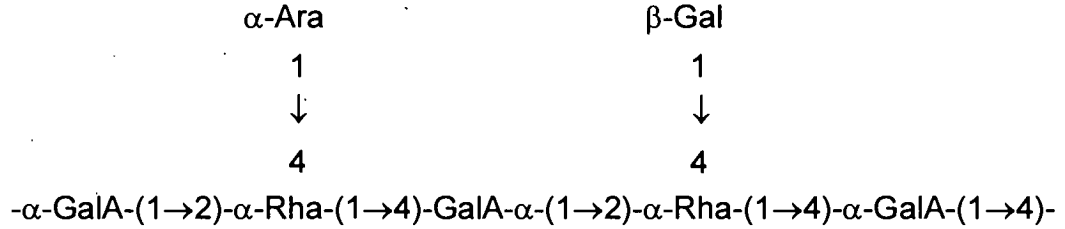


Fig. 1.7: Partial structure of rhamnogalacturonan I.

The pectins can be extracted from cell walls with mild acid or by treatment with hot ammonium oxalate. The middle lamella is primarily composed of pectins. Grass pectin was found to be quite similar to that of dicots but there is a higher degree of branching at the rhamnose units (Carpita, 1989). Bolwell (1993) postulated that the role of pectins was to abstract water from the cellulose — hemicellulose interaction in order to promote their association.

Pectins (especially methyl-esterified homogalacturonans and rhamno-galacturonans) are quantitatively dominant over arabinoglucurono-xylans in dicots; in growing grass cell walls the reverse was found to be the case (Fry, 1989).

1.3.2.3 Glycoproteins

Most cell wall proteins studied to date were found to be glycoproteins (Fry, 1991). The best known glycoprotein is extensin (Fig. 1.8). It is a strongly basic glycoprotein made up of a highly unusual range of monomers: 50% arabinose, 20% hydroxyproline, 6% serine, 5% tyrosine, 5% lysine and other amino acids (O'Neil & Selvendran, 1980). Approximately 40% of its amino acids are hydroxyproline (Brett & Waldron, 1990; Fry, 1991) which are part of a sequence Ser - $(Hyp)_4$. Tri- and tetra-arabinose oligosaccharides are attached to the hydroxyproline residues and single α-D-galactose residues to serine:

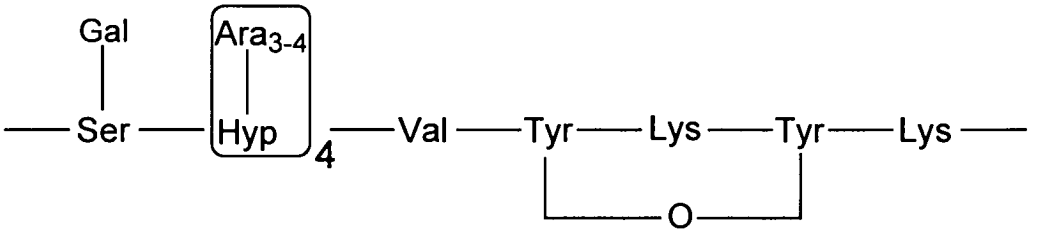


Fig. 1.8: Partial structure of extensin. An intramolecular loop is shown.

Newly synthesised extensin contains 10 mol-% tyrosine (Epstein & Lamport, 1984, Fry, 1985). At this stage it is soluble and can be secreted through the plasmalemma like most of the polysaccharides and glycoproteins

of the wall matrix (Hanke & Northcote, 1974). Extensin binds ionically to the pectic polysaccharides of the cell wall. Later the binding is strengthened, probably by forming intermolecular crosslinks of tyrosine residues (Fry, 1982; Smith *et al.*, 1984; Biggs & Fry, 1990) designated isodityrosine: an oxidatively coupled dimer (Fig. 1.9) of two tyrosine residues with a diphenyl ether bridge. An oxidatively coupled product with four tyrosine residues has been identified recently (Brady *et al.*, 1996).

Epstein & Lamport (1984) have demonstrated an intra-polypeptide isodityrosine linkage (Fig. 1.8). However, it has been suggested that an inter-polypeptide isodityrosine crosslink is also possible (Biggs & Fry, 1987).

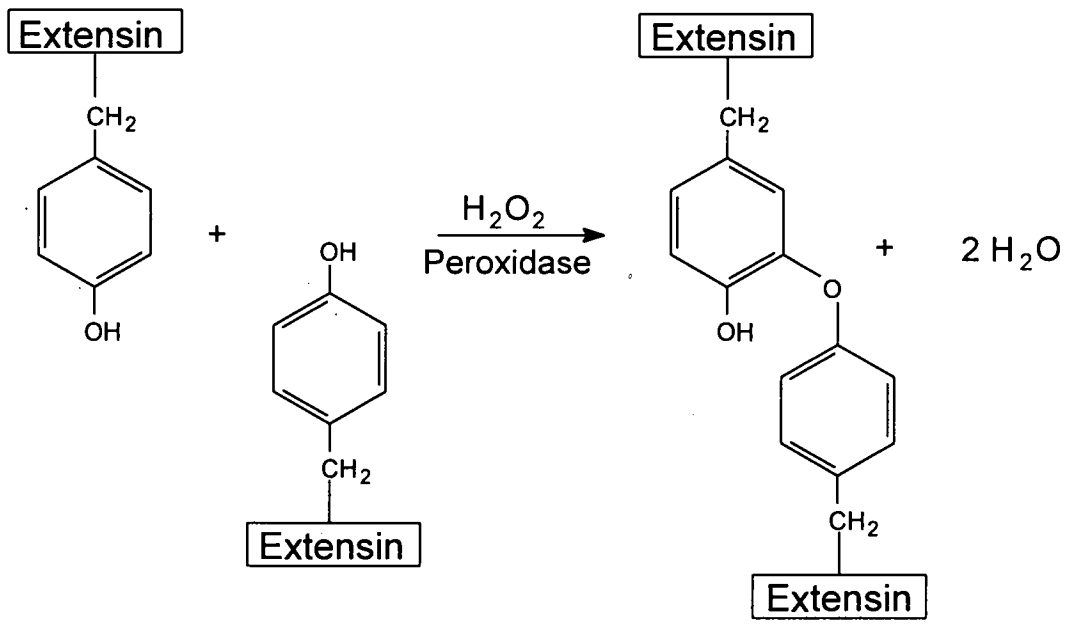


Fig. 1.9: Inter-polypeptide crosslinks with two tyrosine residues to form isodityrosine.

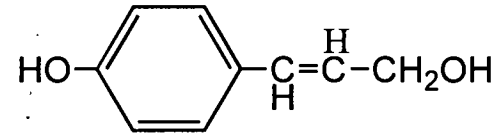
Fry (1982 and 1985) suggested that isodityrosine crosslinks are responsible for the inextractability of cell wall glycoproteins. It is interesting to mention that none of the oxidation-products such as dityrosine, trityrosine, isotrityrosine or dihydroxyphenylalanine known in animals were found in plants (Fry, 1982). Dityrosine and its higher homologues were found in egg shells and cyst walls of plant-parasitic nematodes (Lopez-Llorca & Fry, 1989). The same authors suggested a similarity between nematode egg shells and plant cell walls since both play a role in providing protection against external hazards.

1.3.2.4 Phenolics

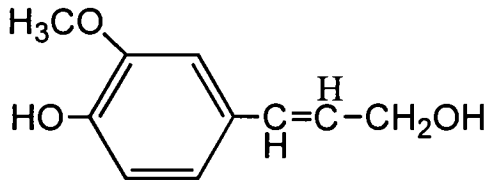
Wall phenolics other than tyrosine derive from the central phenylpropanoid pathway, the first enzyme, phenylalanine ammonia-lyase (PAL, the committed step) leading to cinnamate, from which a wide variety of cinnamate derivatives such as ferulic acid, flavonoids, stilbenoids and lignin are formed.

1.3.2.4.1 Lignin biosynthesis

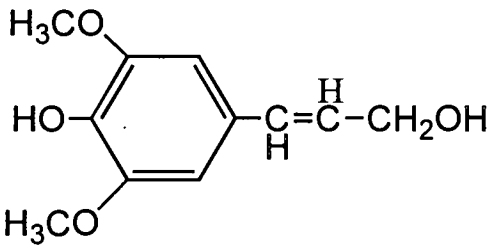
The starting point of lignin biosynthesis is cinnamic acid (Hartley & Jones, 1975, Fry, 1983). *p*-Coumaric, ferulic, and sinapic acids are formed in step by step reactions of hydroxylation and *O*-methylation. Specific monooxygenases are involved in the hydroxylation reactions. The acids are activated by a *p*-coumarate CoA ligase to form their CoA thiol esters: *p*-coumaroyl-CoA, feruloyl-CoA and sinapoyl-CoA. These activated acids are reduced in two steps to their corresponding alcohols (by a cinnamoyl-CoA : NADPH reductase and a cinnamoyl aldehyde : NADPH reductase). For the biosynthesis of lignin, these soluble precursors, namely *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 1.10), undergo repeated oxidative coupling by the action of peroxidase.



p-Coumaryl alcohol



Coniferyl alcohol



Sinapyl alcohol

Fig. 1.10: Precursors for lignin biosynthesis.

The oxidation system (peroxidase and hydrogen peroxide and/or laccase and O_2) removes a single H-atom to form a free radical (Fry, 1989) which undergoes rapid mesomerization (similar to that which may occur in the formation of isodityrosine and diferulate). Polymerisation takes place after cell elongation has come to an end (Brett & Waldron, 1990) by depositing new material and by displacing water as long as enough space is available in the wall. A very strong hydrophobic meshwork (Brett & Waldron, 1990) is built which restricts growth and digestibility. In the extreme case, the cell will die and thereby provide an efficient protection against infection of the plant.

The occurrence of the lignin precursors can be related to different taxa: guaiacyl units (derived from coniferyl alcohol) are dominant in gymnosperm lignin, while angiosperm lignin contains similar amounts of guaiacyl and syringyl units. All three units are found roughly in equal amounts in monocot lignin (Brett & Waldron, 1990).

Since lignin is a large and insoluble molecule and therefore immobile, it must be synthesised at its final destination (Fry, 1985, Bolwell, 1988) from precursors which are synthesised in the cytoplasm. Predominantly diphenyl-ether and biphenyl linkages are formed. The C_3 ("propene") unit also participates in cross-linking (Wallace & Fry, 1995).

The heteropolymer net of lignin is more complex in monocots where in addition *p*-coumaric and ferulic acids are ether-linked (Bolwell,1993) to the net and further esterified to arabinoxylans (Lewis & Yamamoto, 1990).

1.3.2.4.2 Hydroxycinnamic acids

It is well established that hydroxycinnamic acids are present in land plants as soluble esters as well as insoluble esters (cell wall bound) (Harborne & Corner, 1961; Harris & Hartley, 1980; Hartley & Harris, 1981). The most common hydroxycinnamic acids found in cell walls are ferulic and *p*-coumaric acids (Yamamoto *et al.,* 1989). It has been demonstrated that these acids are released from grass cell walls by treatment with alkali (Brown, 1966). These compounds could be shown to be present in angiosperms, particularly those belonging to the Gramineae (Smith, 1955) such as Timothy grass and wheat (Stafford, 1962; El-Basyouni & Towers, 1964; Geissmann & Neukom, 1973). It has since been discovered that the hydroxycinnamic acids are linked to arabinoxylans of monocots (Ahluwalia &

Fry, 1986; Kato & Nevins, 1985; Gubler *et al.*, 1985). Fry (1982) and Fry & Miller (1989) found feruloyl groups in cell walls of dicots (spinach) attached to arabinose and galactose residues of pectic polysaccharides. These phenolic compounds are distinctly different from lignin (Fry, 1984).

Both ferulic and *p*-coumaric acids are predominantly present in their more stable *E*-isomeric form (Hartley & Jones, 1976, Hartley, Jones & Wood, 1976, Gubler *et al.*, 1985, Yamamoto, Bokelman & Lewis, 1989). It has been suggested that photoisomerism of cell wall bound hydroxycinnamoyl esters is responsible for growth changes (Yamamoto & Towers, 1985). Irradiation with blue light has been shown to inhibit growth (Cosgrove, 1981). UV-A light leads to *E/Z*-isomerisation (Towers & Abeysekera, 1984) (Fig. 1.11). This is a reversible phenomenon and depends on the irradiation wavelength.

$$\text{(}E\text{)-Hydroxycinnamic esters} \xleftrightarrow{\text{UV-A}} \text{(}Z\text{)-Hydroxycinnamic esters}$$

For this reaction to be driven by blue light, which is not absorbed by hydroxycinnamoyl esters, a photoreceptor is required; it could be either a carotenoid or a flavin:

$$\text{Photoreceptor} \xrightarrow{\text{blue light}} \text{Photoreceptor *}$$

The subsequent reaction can be given as:

$$\text{Photoreceptor *} + \text{(}E\text{)-Hydroxycinnamic esters} \longrightarrow \text{Photoreceptor} + \text{(}Z\text{)-Hydroxycinnamic esters}$$

Fig. 1.11: Photoisomerisation of cell wall bound hydroxycinnamic acids. * = triplet state (Towers & Abeysekera, 1984).

It has been reported that grass cell walls (e.g. *Lolium*, *Phleum* & barley) contain both *E*- and *Z*-isomeric forms of hydroxycinnamoyl esters (Hartley & Jones, 1977, Yamamoto & Towers, 1985). However, *Z*-isomers could not be detected in tissues grown in the dark but only after exposure to UV-A light (320 - 400 nm). This indicates an *E/Z* - isomerisation of hydroxycinnamic acids *in vivo*.

Alkaline hydrolysis of arabinoxylans released ferulic acid, which had been esterified to arabinose side chains (Smith & Hartley, 1983). By measuring the UV absorption of cell walls of grasses, Hartley *et al.* (1973) found a characteristic maximum at 324 nm. This characteristic UV absorption maximum is very similar to authentic ferulic acid (Fincher, 1975). After addition of alkali a large shift to approximately 370 nm can be seen (Gowda & Sarathy, 1987). The feruloyl groups show at neutral pH an intense blue fluorescence under UV light (366 nm) turning to blue-green under ammonia vapour (Fry, 1986) because of the ionisation of the phenolic hydroxyl group. Feruloyl esters can be differentiated from *p*-coumaric esters since the latter do not fluoresce without ammonia and have higher R_f-values (Fry, 1982). Morrison (1977) and Ishii (1991) reported on an arabinoxylan being esterified by ferulic acid and by acetic acid (at positions *O*-5 and *O*-2, respectively). MacKenzie *et al.* (1987) found both ferulic and *p*-coumaric acid esterified in barley straw cell walls.

The formation of feruloyl esters seems to be highly specific: ferulic acid is attached very predominantly to position *O*-5 of L-arabinofuranosyl side chains of arabinoxylans (Smith & Hartley, 1983; Kato & Nevins, 1985; Nevins & Kato, 1985; MacKenzie *et al.*, 1987; Hatfield *et al.*, 1991; Ishii, 1991). Fractionation of barley endosperm cell walls into their β-glucan and their arabinoxylan showed that all the ferulate groups were carried by the arabinoxylan (Ahluwalia & Fry, 1986).

It appears that there is a significant difference between monocots and dicots in terms of the specificity of the ferulic ester linkage (Kato & Nevins, 1985). Fry (1982) identified an esterification at position *O*-6 of galactose and at position *O*-3 of arabinose in the pectic polysaccharides of suspension-cultured spinach cells.

1.4 Formation of diferulic crosslinks causes gelation

It has been observed that the water-soluble arabinoxylans are susceptible to gelation in the presence of oxidising agents (Amadò & Neukom, 1985). It was concluded (as in extensin) that the arabinoxylans must contain groups which are very sensitive to oxidation (Amadò & Neukom, 1985; Ahluwalia & Fry, 1986). Such a group is ferulic acid (Harris & Hartley, 1980; Tanner & Morrison, 1983; Gubler & Ashford, 1985).

The cause of the gelation was found to be the formation of diferulic acid (Geissmann & Neukom, 1973; Fry, 1984; Shibuya, 1984; Amadò & Neukom, 1985; Markwalder & Neukom, 1976) (Fig. 1.12).

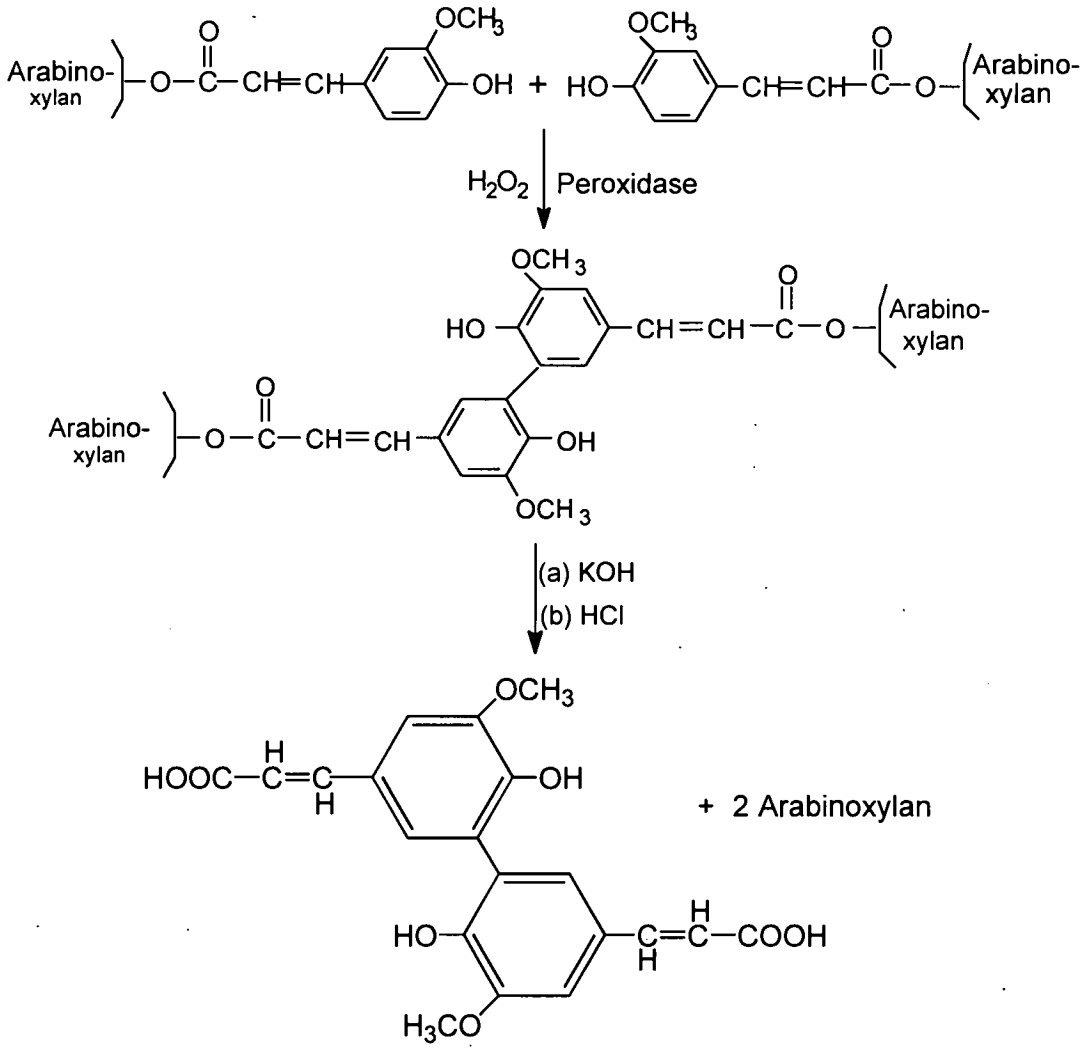


Fig. 1.12: Formation of diferulic acid crosslinks by oxidative phenolic coupling of ferulic acid residues in arabinoxylans (after Markwalder & Neukom, 1976).

The first step in the sequence of reactions is the formation of free radicals (Fig. 1.13) by removal of a single H atom in the presence of peroxidase and hydrogen peroxide (similar to the formation of isodityrosine and to lignin biosynthesis). The radicals are highly reactive. They undergo tautomerisation and can react with each other to form biphenyls or diphenyl

ethers (Fry, 1983). Such biphenyls and diphenyl ether bonds are highly stable under biological conditions. These dimers, once created, can further oxidised to yield larger oligomers (Fry, 1986). However, Ralph *et al.* (1994) propose other types of cross-links as well.

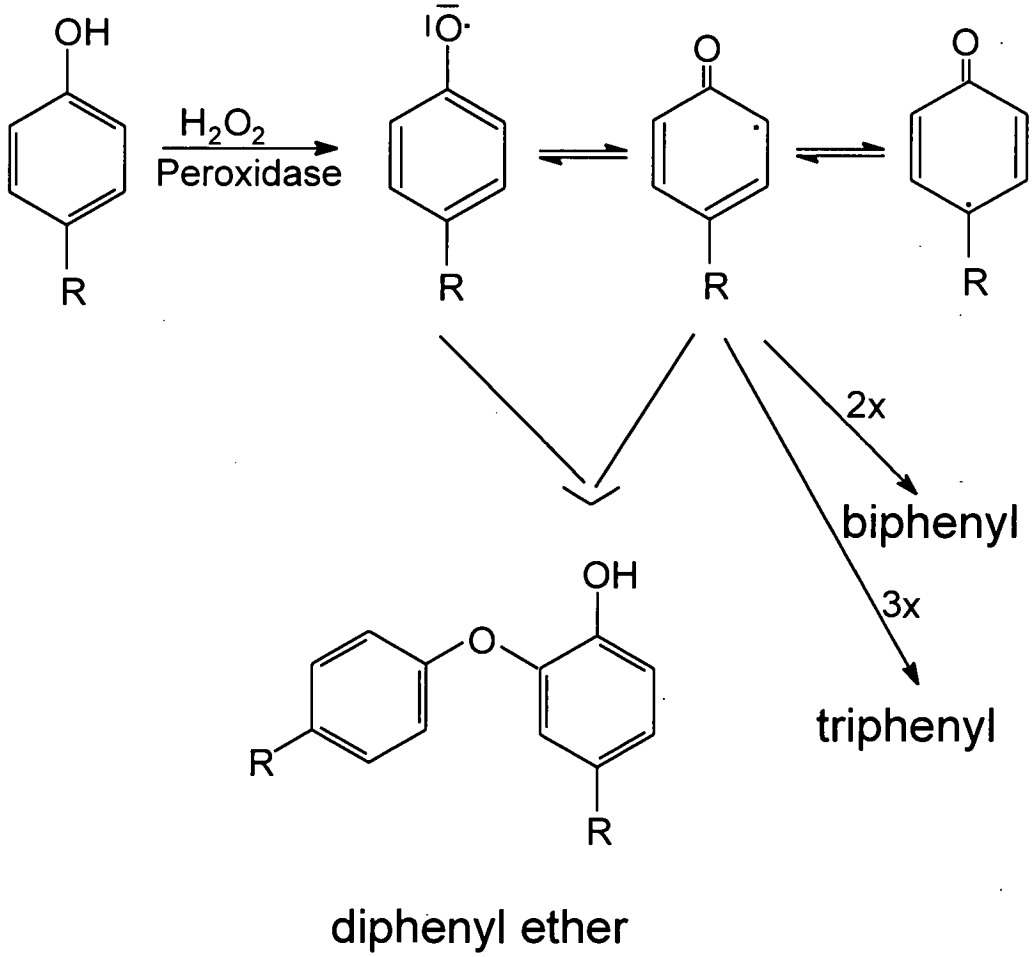


Fig. 1.13: General formation of diphenyl ethers, biphenyls and larger oligomers (see appendix).

Markwalder & Neukom (1976) presented evidence that diferulate crosslinks insolubilize arabinoxylans. It appears that the formation of diferulic acid is more specific (however this view has been contradicted recently by Ralph *et al.*, 1994) (as is true for the formation of isodityrosine) in comparison to lignin biosynthesis where a heterogeneous mixture of diphenyl and ether bonds are generated. In addition, it was observed that the gel obtained after oxidative coupling could be liquefied by proteolytic enzymes

(Amadò & Neukom, 1985). It was suggested that proteins must be involved in the formation of the gel as well (Fig. 1.14) (Neukom & Markwalder, 1978, Amadò & Neukom, 1985) to form a tyrosine-ferulic acid network.

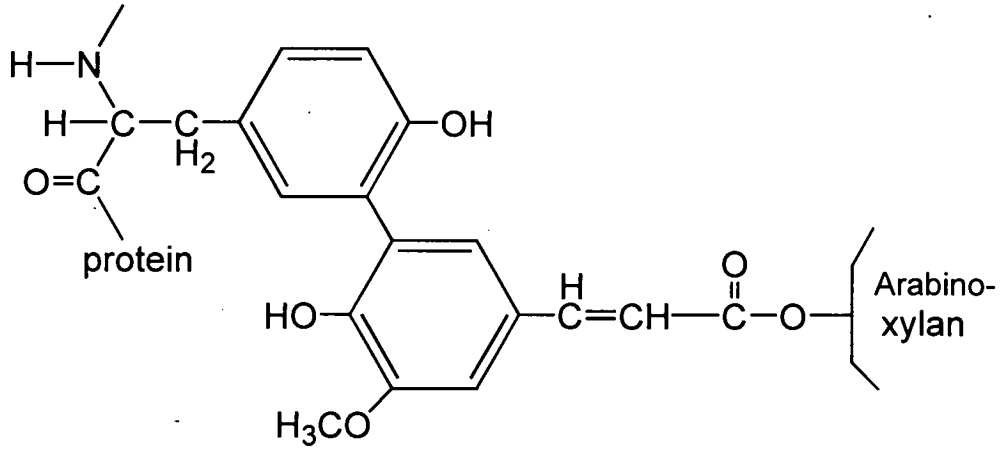


Fig. 1.14: Hypothetical cross-linking of arabinoxylans containing ferulic acid and proteins.

Peroxidase-catalysed oxidative coupling can be seen as a general method for strengthening the structure of the growing cell wall (Fry, 1986) since a high degree of crosslinks will tighten the cell wall and a low degree will keep it loose.

1.5 Suspension cultured cells

To study the primary cell wall, many scientists take advantage of suspension cultured cells. Zimmermann & Hahlbrock (1975) purified phenylalanine ammonia lyase (PAL) - probably the most studied enzyme of secondary metabolism (Bolwell, 1993) - from cultured parsley cells. The PAL has been studied in tobacco and carrot cell lines as well (Berlin & Widholm, 1978). Suspension cultured cells are easy to handle and offer a homogeneous source (uniform state) of primary walls without the presence of secondary walls. They can be grown on a large scale without the presence of micro-organisms, and can be obtained in a relatively short period of time. The doubling time of the fastest-growing plant cell suspension culture is

approximately 20 h (cultured cells of fescue come close to this doubling time); the average doubling time is about 45 hours (Ludden & Carlson, 1980).

One should question whether the data obtained from such a source could be relevant to intact tissues. Plant cell cultures are not just single plant cells (like bacteria); they grow in small clumps of cells. In sycamore and Paul's scarlet rose cells (very finely divided cultures), approximately 30% of the cells may remain in clumps with fewer than ten cells (Ludden & Carlson, 1980). Nevertheless, the tissue complexity (in comparison to tissue from a whole plant) is reduced to a minimum.

Table 1.2 shows the compositions of monocot primary cell walls obtained from cell cultures and from plant tissues. It is apparent that the primary cell walls from suspension cultured cells are quite similar to those from intact monocot tissues (Darvill *et al.*, 1980). Methylation analysis of primary cell walls gave further evidence for this (Burke *et al.*, 1974). The same is true for primary walls of dicots.

Component	Components of cell walls (%) isolated from				
	Intact tissues		Suspension cultured cells		
	Coleoptile	Internode			
	Maize	Oat	Oat	Wheat	Rice
Glucose	30.5	40.0	14.9	17.9	16.3
Rhamnose	Tr	0.4	1.0	0.6	2.0
Fucose	Tr	Tr	1.5	0.1	1.0
Arabinose	20.0	12.0	19.4	20.7	27.1
Xylose	17.6	18.9	21.5	24.8	17.1
Mannose	1.7	3.0	0.8	1.0	0.3
Galactose	5.4	2.0	12.5	12.0	7.0
Uronic acids	8.9	17.0	11.0	12.0	18.0
Protein	12.9	6.5	16.0	11.0	17.0
Hydroxyproline	3.0	0	0	0.14	0.13

Table 1.2: Comparison of the compositions of monocot primary cell walls obtained from plant tissues and from suspension cultured cells in % (after Darvill *et al.*, 1980); Tr = trace.

Although there are some limits to the use of suspension cultured cells, this experimental system offers some distinct advantages (e.g. no diffusion barriers, no diverse cell types, and no differential response to plant hormones - Ludden & Carlson, 1980) to plant physiologists.

Despite the fact that there are variations among cells derived from suspension cultures, great success has been achieved using suspension-cultured cells as an experimental system.

1.6 The aim of the project

The roles of ferulic acid esterified to carbohydrates have not been completely elucidated (Hatfield *et al.,* 1991). Numerous functions have been proposed. A correlation has been reported between the release of ferulic acid after alkaline hydrolysis and resistance to microbial degradation (Jung & Fahey, 1981). It could be shown that ferulic acid inhibited coleoptile elongation (Tan *et al.,* 1992). Fry (1986) proposed a crosslinkage of feruloylated polysaccharides by forming diferulic acid to restrict cell wall extensibility.

Recently it has been reported (Myton, 1993) that feruloylated arabinoxylans of cultured *Festuca* cells are relatively resistant to the enzyme mixture Driselase whereas Driselase can act successfully on other graminaceous cell walls (e.g. maize and barley) to release feruloylated oligosaccharides such as Fer-Ara-Xyl (FAX) and Fer-Ara-Xyl_2 (FAXX).

The resistance of *Festuca* feruloyl-arabinoxylans to Driselase means that only a very low percentage of the feruloyl groups were released as FAX and FAXX. The feruloylated material was solubilised by Driselase but in the form of relatively high molecular weight conjugates. The question of why *Festuca* feruloyl-arabinoxylan cannot be digested by Driselase to small fragments such as FAX and FAXX to any great extent is investigated in the present thesis.

The main objectives of the work described in this thesis are:

(1) Investigation of the structure of compounds released from cell walls of fescue, by mild acid hydrolysis and Driselase hydrolysis.

The analysis concentrates on relatively low-molecular weight conjugates and involves the characterisation of the sugar composition, the reducing terminus, the glycosidic linkages involved (within a particular conjugate as well as its linkage to the xylan backbone) and the attachment of ester groups. In a comparison, the percentage of Driselase-solubilised products released from fescue and maize AIR will be determined as well as the percentage of solubilised material that is oligomeric rather than polymeric.

(2) To find out at which stage in the *in vivo* biosynthesis of a feruloylated arabinoxylan the feruloylation of the arabinose takes place: before or after a xylosyl residue is attached.

(3) To reveal whether or not rat caecal bacteria are able to degradate plant cell wall fragments such as compound B.

(4) Investigation of whether the major compound released after mild acid hydrolysis (compound B) is unique to *Festuca arundinacea* or is more widespread.

2. Material and Methods

2.1 Plant material

2.1.1 Cell suspension cultures

Cell suspension cultures of tall fescue grass (*Festuca arundinacea* Schreber) and rose (*Rosa* sp., cv. Paul's Scarlet) were used as experimental material.

Fescue cultures were incubated at 25°C with photon flux density of 20 μmol $m^{-2}s^{-1}$ and rotated in a horizontal plane at 110 rpm with an amplitude of 3.5 cm. Rose cultures were incubated at 25°C with photon flux density of 2.5 μ mol $m^{-2}s^{-1}$ and rotation in a horizontal plane at 100 rpm and an amplitude of 1.5 cm.

All media and glassware used in the maintenance and manipulation of cell suspension cultures were sterilised by autoclaving at 121°C for 18 min. Aseptic manipulations of cultures were carried out in a laminar flow cabinet.

In order to prevent contamination, the flasks containing the culture suspension were sealed with sterile cotton wool bungs and covered with aluminium foil.

Both cultures were sub-cultured into fresh medium (see appendix) every two weeks.

2.1.2 Other plant material

Other material worked with were cucumber seeds (Masterpiece, Wm.K. McNair, Portobello, Edinburgh) and seeds of *Festuca arundinacea*, *Festuca gigantea*, *Festuca pratensis*, *Festuca rubra*, *Lolium multiflorum*, *Lolium perenne*, *Dactylis glomerata, Bromus sterilis, Agrostis tenuis* and *Phleum pratense* (§ 3.12) (kindly donated by Dr K.J. Webb, IGER, Aberystwyth, Dyfed, Wales). *Sorghum verticilliflorum, Dichanthium sericeum* and *Bothriochloa ambigua* were a gift from Moffat Pinkie Setshogo (Edinburgh University). Seeds of *Secale sentinal*, *Sorghum vulgare* and *Hordeum vulgare* were obtained from Wm.K. McNair, Portobello, Edinburgh. All plantlets were grown in the greenhouse at Edinburgh University and were harvested after 6 weeks. *Triticum aestivum, Avena sativa, Arrhenatherum elatius, Anthoxanthum odoratum* and *Trachycarpus fortunei* were collected in Edinburgh. *Zea mays* was collected in Santiago de Compostela, Spain).

2.2 Other material

Fresh caecal content was taken from a rat (Wistar, male, 300 g body weight) for an investigation as to whether rat caecal bacteria are able to degrade compound B (§ 3.11) or not.

For an initial study, approximately 5 ml caecal content was made up to 10 ml with Scott and Dehority medium (see appendix) and mixed with [^{3}H]arabinose (6 MBq). All manipulations of medium and gut contents were performed under N_2. Samples of the fermentation (1.02 ml) were added to 3 ml of methanol/formic acid (4 : 1) at t=0, 5, 10, 20, 30, 40, 50, 60 and 120 min. The sample for t=0 was mixed with 3 ml methanol/formic acid first and then [^{3}H]arabinose (1/10) was added. The time of incubation was changed later to t=0,½ , 1, 1½ , 2, 4, 8, 16, 32 and 64 min.

For the investigation of the degradation of compound B, 40 kBq of this compound (^{3}H-labelled compound B in the first and ^{14}C-labelled compound B in the second experiment) was mixed with gut content and treated as described above.

2.3 Chemicals

2.3.1 General chemicals

All chemicals used were obtained from either Sigma Chemical Co. or BDH Chemical Ltd. and were of AnalaR quality when available. The water used was singly distilled in a glass still.

For paper electrophoresis markers (§ 3.5.7.1) β-D-galactosyl-(1→3)-D-arabinose and 2-deoxy-D-ribose were reduced with $NaBH_4$ (§ 2.6.6) to yield β-D-galactosyl-(1→3)-D-arabinitol and 2-deoxy-D-ribitol (≡2-deoxy-D-arabinitol), respectively.

2.3.2 Radiochemicals

2.3.2.1 L-[1-^{3}H]Arabinose

L-[1-^{3}H]Arabinose was obtained from Amersham International Plc., produced by the T - 7 technique: L-arabinose is treated with $^3H_2$ in the presence of a catalyst (Evans *et al.,* 1974). By this treatment the H atom at

the reducing terminus will be replaced by tritium. The specific activity of the L-[1-^{3}H]arabinose was 97 TBq/mol.

2.3.2.2 [^{3}H]Xylobiose and [^{3}H]isoprimeverose

[^{3}H]Xylobiose and [^{3}H]isoprimeverose were prepared from rose cell suspension cultures (Fry, 1988). A suspension culture of rose (*Rosa* sp., cv Paul's scarlet) (20 ml, 16 days after sub-culture) was incubated under standard conditions with 18 MBq of L-[1-^{3}H]arabinose for 6 h. The cells were washed several times with 80% ethanol, passed through a Büchner funnel and washed again with 80% ethanol. A final wash with acetone was applied. To digest the AIR (10 mg), 10 mg Driselase in 2.4 ml buffer (§ 2.6.3.1.1) was used for 27 hours. The hydrolysis was stopped with 800 µl formic acid and the suspension centrifuged for 10 min at 2500 rpm. The supernatant was dried *in vacuo* in a Speed-Vac, subjected to preparative paper chromatography on Whatman 3MM as a streak (30 cm) and developed in EPW_2 (§ 2.5.1) for 28 h. Xylose, xylobiose and isoprimeverose were applied as non-radioactive external markers, which were cut off and stained (§ 2.8.1).

To detect the radioactivity on the chromatogram, the paper was dipped in 7% PPO in diethyl ether, dried and fluorographed (§ 2.4) (Fry, 1988) for 23 days. After developing the film, the regions of the paper including [^{3}H]xylobiose and [^{3}H]isoprimeverose were cut out individually, and the material was eluted with water and dried *in vacuo* in a Speed-Vac. The compounds were dissolved in 2 ml water (each) and a small part (1µl) was assayed for radioactivity (§ 2.7.1.2).

2.4 Equipment

The paper for chromatography (PC) and electrophoresis (PE) was supplied by Whatman International Ltd.

High voltage paper electrophoresis (HVPE) was performed on a flatbed, water-cooled system from Shandon with a high voltage power pack from Savant Instruments Inc.

Samples were dried *in vacuo* with a Speed-Vac centrifugal evaporator from Savant Instruments Inc. or a Thermostat vacuum oven from Townson & Merzer Ltd. A LSC Secfroio Freeze-Drier was used to freeze-dry samples.

Centrifugation was carried out on a Centaur 2 (at 2500 rpm = 550 g), a Micro Centaur (at high speed), a Mistral 4C (at 3400 rpm = 1200 g) (all three from MSE) and on a Sorvall RC-5B Refrigerated Superspeed Centrifuge (at 12500 rpm = 30000 g).

The shaker used (§ 2.6.4) was an orbital incubator from Gallenkamp (England) at 175 rev/min.

Radioactive samples were assayed on a Beckman LS 5000 CE scintillation counter.

X-ray films used for fluorography were Du Pont Cronex 4 Pelicula (30 cm x 40 cm). The fluorograms were developed in an automatic film processor Compact X2 from X-Ograph Ltd. to show the position of radioactive zones (§ 2.3.2.2).

Spectrophotometric studies were performed on a DU-64 Spectrophotometer from Beckman.

High pressure liquid chromatography (HPLC) was performed on a Gilson, using:

* 2 piston pumps, Model 302
* sample injector, Model 231
* manometric module, 802C
* dynamic mixer, 811
* dilutor 401
* fraction collector, Microcol TDC 80
* UV spectrophotometric detector, Shimadzu SPD-6A
* fluorescence HPLC monitor, Shimadzu RF-530
* plotter, Shimadzu Chromatopac C-R3A
* HPLC System Manager Model 702, Version 3.0

Products released after methylation analysis of compound B were identified by GC-MS on an SSQ 710 spectrometer equipped with an SP 2330 column (Institute of Chemistry, Bratislava, Slovakia).

An FT-IR spectrum of compound B as a film on an NaCl plate was recorded on an FTIR Nicolet Magna 750 spectrometer (Institute of Chemistry, Bratislava, Slovakia).

NMR-spectra (^{1}H and ^{13}C) of compound B were acquired on an FMTIR spectrometer Bruker AM 300 (Institute of Chemistry, Bratislava, Slovakia).

2.5 Fractionation methods

2.5.1 Paper chromatography (PC)

Samples were applied to Whatman 3MM chromatography paper (46 x 57 cm) either as spots or as streaks (9 cm from the end). Sugars as standards were loaded in the range of 50-100 μg. Chromatograms were developed by descending chromatography in the following solvent systems. The one with the best properties in the specific case (Fry, 1988) was chosen:

BAW, butan-1-ol/acetic acid/water (12:3:5, v/v/v), run for 18 h;
BEW, butan-1-ol/ethanol/water (20:5:11, v/v/v), run for 16 h;
BPW, butan-1-ol/pyridine/water (4:3:4, v/v/v), run for 18 h;
BAB, butanone/acetic acid/boric acid (sat. in water) (9:1:1, v/v/v), run for 30 h;
EAW, ethyl acetate/acetic acid/water (10:5:6, v/v/v), run for 9-16 h;
EPW, ethyl acetate/pyridine/water (8:2:1, v/v/v), run for 6.25-48 h;
EPW, ethyl acetate/pyridine/water (10:4:3,v/v/v), run for 28 h;
ΦW, 80% w/w in water, run for 20h.

The position of a compound on a paper chromatogram is given as an R_f-value, i.e. the position of a specific compound relative to the solvent front:

$$R_f = \frac{\text{distance of a compound from origin}}{\text{distance of solvent front from origin}}$$

or as an R_{Ara}-value, i.e. the position of a specific compound relative to the standard arabinose:

$$R_{Ara} = \frac{\text{distance of a compound from origin}}{\text{distance of arabinose from origin}}$$

2.5.2 High voltage paper electrophoresis (HVPE)

High voltage paper electrophoresis was generally performed at 2000 V. Samples were loaded on to chromatography paper and separated by electrophoresis in various buffers (200 ml in each trough):

(a) molybdate buffer: pH 2,3 and 4 (adjusted with formic acid) for 5 h
(MoO_4^{2-}) pH 5 (adjusted with H_2SO_4) for 2.5 h
(b) borate buffer: pH 9.4 (adjusted with NaOH) for 1.67 h to 2.5 h
($B_4O_7^{2-}$)

For (a), 2% aqueous sodium molybdate was used and for (b) 1.9% aqueous sodium borate.

The basis of this technique is that the anions bind to the neutral polyhydroxy compounds and give them a negative charge and thus electrophoretic mobility (Weigel, 1963). Bromophenol blue (blue) and picric acid (yellow) were used as visible markers.

2.5.3 High pressure liquid chromatography (HPLC)

Compound B was prepared from *Festuca* AIR (25 g) by mild acid hydrolysis, pre-purified by reverse-phase column chromatography (Mega RPC), PC in BAW followed by PC in BEW, and further purified by HPLC (for NMR studies, § 3.5.10) by using an analytical reversed-phase (C_{18}) column (25 cm x 4.6 mm i.d. column of Spherisorb S50D2 from Hichrom) at 25°C. The material together with a small portion of ^{14}C-labelled compound B was eluted with a H_2O/MeCN-gradient of 20→50% for 40 min at 1 ml/min. The fluorescence of the eluant was scanned and the absorbance at 280 nm monitored (280 nm was chosen - instead of the absorption's maximum at 323 nm for feruloyl esters - to detect most of the impurities). Fractions were collected (40 x 1 ml) and assayed for radioactivity. In later work, the gradient was optimised to an H_2O/MeCN-gradient of 15→25%.

Based on this result, a preparative reverse-phase HPLC column (25 cm x 10 mm i.d. of Spherisorb S50D2 from Hichrom) was used under the same condition as described above, but with a flow rate of 4 ml/min. Fractions containing compound B were collected and dried *in vacuo* for NMR studies and methylation analysis (§§ 3.5.10 and 3.5.11).

Pre-purified compounds A and B from 14 different grasses (§§ 2.6.9.2 and 3.12.2.2) were applied to an analytical reversed-phase HPLC column and the material eluted with a H_2O/MeCN-gradient of 15→25% for 30 min at 1 ml/min flow rate. The fluorescence and absorbance were monitored as mentioned above.

2.5.4 Spectroscopy

2.5.4.1 FT-IR spectroscopy

An IR spectrum of purified compound B on an NaCl plate was recorded between 4000 and 600 cm^{-1} by 128 scans.

2.5.4.2 NMR spectroscopy

NMR spectra of purified compound B (3.5 mg) were acquired at 25°C in D_2O in a 5 mm sample tube and 10 mm multinuclear NMR probe (^1H: 300.13 MHz and ^{13}C: 75.46 MHz).

2.5.5 Gel permeation chromatography (GPC); Bio-Gel P-2

Samples were passed through a column of Bio-Gel P-2 (bed volume 180 ml) together with non-radioactive markers (xylose, xylobiose, glucose, maltose and maltotriose; each 2 mg) and were eluted with HOAc/Pyr/water (1:1:23 by volume, pH ~4.7) at a flow rate of 6.8 ml/h. Fractions (80 x 2.6 ml) were collected and aliquots used for further investigation:

(a) 100 µl was assayed for hexose;

(b) 100 µl of fractions containing hexoses were spotted onto paper and developed in BAW (§ 2.5.1) with marker sugars which were located by staining with aniline hydrogen phthalate (§ 2.8.1);

(c) 2 ml was assayed for radioactivity (§ 2.7.1.2).

2.5.6 Gel permeation chromatography (GPC); Sephadex G-25

A 1100-ml bed volume column of Sephadex G-25 was used for partial purification of Driselase (§ 2.6.3.1.2). After precipitation with 52% (w/v) ammonium sulphate and centrifugation, the pellet was dissolved in water and loaded on to the column. Water was chosen as eluant (§ 2.6.3.1.1).

2.5.7 Ion-exchange chromatography

2.5.7.1 Cation-exchange on Dowex-50W

A 1.5-ml bed volume column (Polyprep) of Dowex 50W (50X8-200) was washed with 2 ml 1 M HCl followed by 20 ml water. This column was used for removing sodium and ammonium ions after reduction with sodium borohydride (§ 2.6.6). Routinely, a dried sample (e.g. a solution of 100 to 200 µl of compound B) that had been incubated 2x with 200 µl 0.5 M $NaBH_4$ in 1 M NH_3 and then made acidic with 80 µl glacial acetic acid was applied on to the column. The unbound (= non-cationic) material was eluted with 8 ml water.

2.5.7.2 Anion-exchange

2.5.7.2.1 Dowex-1

A 3-ml bed volume column (Polyprep) of Dowex 1 (1X4-200) was washed with 6 ml 2 M NaOH and equilibrated with 50 ml water. Samples from the Dowex-50W column (§ 2.5.6.1) were dried to remove volatile acids (acetic and boric), then re-dissolved in 1 ml of water and applied to a Dowex-1 column to remove iodate ions (IO_3^-). The unbound (= neutral) material was eluted with 8 ml water.

2.5.7.2.2 DEAE-Sephadex A-50

A 2-ml bed volume column of DEAE-Sephadex A-50 in 10 mM acetic acid, pH 4.7 (adjusted with pyridine) was used. β-Xylosidase solution (commercial sample from Sigma Chemical Co., after dialysis, § 2.6.3.2) was applied to the column and eluted with a gradient of acetate (10 mM to 1 M, Pyr^+ salt, pH 4.7) and 2-ml fractions (30) were collected. Each of the fractions was assayed for β-xylosidase (§ 2.7.4) with methyl β-xyloside as substrate and for α-xylosidase with methyl α-xyloside and [^{3}H]isoprimeverose as substrates.

2.6 Analytical methods for cell wall-bound polysaccharides

2.6.1 Mild and complete acid hydrolysis with TFA

TFA is the acid of choice for hydrolysis because it is volatile and for removing the acid no further step is necessary other than to dry the sample *in vacuo*. A sugar (glycosyl residue) is fixed in a polysaccharide in either a furanose-form (5-membered ring) or a pyranose-form (6-membered ring). Pyranose-forms (and feruloyl esters) are much more stable to mild acid hydrolysis than furanose-forms. Knowing this, the first step of releasing feruloylated oligosaccharides from cell walls of fescue (§ 3.1) and other grasses (§ 3.12) was treatment with 0.1 M TFA (1 ml/20 mg dry weight) at 100°C for 1 h. To obtain these optimised conditions, three test series were carried out using the method of changing one parameter while keeping the others constant.

In the first series the temperature was varied from 20°C to 100°C (in 10°C-steps). These hydrolyses were conducted with 0.1 M TFA for 1h. In the second series the time was varied from 10 min to 60 min (in 5 min-steps). These hydrolyses were conducted with 0.1 M TFA at 100°C. In the third series the acid concentration was varied from 0.01 M to 0.1 M TFA (in 0.01 M-steps). These hydrolyses were conducted for 1h at 100°C. In each case 60 mg AIR was hydrolysed with 3 ml TFA. The hydrolysates were dried, dissolved in 40 µl water, divided into two portions, applied on to paper and developed in BAW (one portion) and EAW (the other portion). The results were analysed on the basis of the highest yield on compound B (by visual comparison). Compound B and other feruloylated oligosaccharides (§ 3.1) can be detected by brief exposure to a 366-nm UV-lamp: blue fluorescence at neutral or acidic pH turning to bright blue-green during fuming with ammonia vapour. The colour of the fluorescence (and the change) is a strong indication of the presence of feruloyl esters. It turned out that the highest yield of compound B could be obtained after hydrolysis in 0.1 M TFA at 100°C for 1 hour.

To achieve complete hydrolysis, dried samples were dissolved in 500 µl 2 M TFA and incubated at 120°C for 1 h in a sealed glass tube. After cooling the tubes, acid was removed by drying *in vacuo* on the Speed-Vac. The hydrolysed material was then dissolved in 30 - 50 µl water, applied to chromatography paper and developed in one of the solvents (§ 2.5.1).

2.6.2 Alkaline hydrolysis

To cleave feruloyl ester-linkages, alkaline hydrolysis was performed routinely by adding either 200 µl 0.5 M sodium hydroxide (pH ~13.3) or 200 µl 0.1 M sodium hydroxide (pH ~12.8) to a dry sample and incubated for 1 hour at 25 °C under nitrogen in the dark.

2.6.3 Enzymic hydrolysis

2.6.3.1 Driselase

Driselase (Sigma Chemical Co.) is a mixture of endo- and exo-*O*-glycosyl-hydrolases from the fungus *Polyporus tulipiferae* (*Irpex lacteus*). This enzyme mixture will digest most of the plant cell wall polysaccharides quite effectively to release small fragments such as FAX and FAXX, but not the feruloylated polysaccharides that are present in the cell wall of the tall grass fescue (see introduction and § 3.10).

2.6.3.1.1 Partial purification of Driselase

10 g of the crude commercially available mixture was dissolved in 100 ml 50 mM acetic acid (adjusted to pH 5 with 1 M NaOH) and stirred for 15 min. After centrifugation for 10 min at 2500 g (3400 rpm) the supernatant was collected (97.3 ml) and 26 g ammonium sulphate per 50 ml was added slowly with constant stirring. The solution was kept at 0°C for 15 min and then centrifuged at 3400 rpm (1200 g) for 10 min. The supernatant was rejected and the pellet re-dissolved in 100 ml of fresh 52% (w/v) ammonium sulphate. The precipitate was collected by centrifugation (10 min at 3400 rpm), dissolved in 10 ml water and de-salted on Sephadex G-25 (§ 2.5.5) with water as eluant. The colourless part was discarded and the fast eluting brown material was collected, freeze-dried and stored below 0°C (Fry, 1982).

2.6.3.1.2 Hydrolysis with Driselase

The buffer used for hydrolysis was pyridine/acetic acid/0.5% chlorbutanol (1:1:98, v/v/v), pH 4.7. Routinely, 10 mg dried sample was dissolved in 0.5 ml buffer, containing 2.5 mg Driselase. Enzyme solutions with samples were incubated at 25°C for 48 hours on a shaking incubator.

To test whether compound B is part of other compounds or not, a portion (each) of ^{3}H-labelled compounds C, D and E were treated with Driselase over a time-course for 48 h, 96 h and 144 h (§§ 3.6.5, 3.7.3 and 3.8.3).

2.6.3.2 β-Xylosidase

The β-xylosidase (Sigma) was supplied as a suspension in 3.5 M $(NH_4)_2SO_4$. The suspension was centrifuged in an Eppendorf vial and the supernatant was removed with filter paper. The protein was re-dissolved in 400 µl buffer (2% pyridine, adjusted to pH 5.0 with acetic acid).

The hydrolysis of ^{3}H-labelled compound B (§ 3.5.8.1) was carried out over a time-course. After 10 min, 50 min, 250 min and 1250 min the reaction was stopped with 10 µl formic acid (per 100 µl of the mixture) and the products were subjected to PC in BAW (§ 2.5.1) on Whatman 3MM as a 5-cm streak to accommodate the remaining ammonium sulphate.

To test whether the feruloyl group within compound B is linked to *O*-5 of arabinose, a portion of ^{14}C-labelled compound B was treated with β-xylosidase for 40 min, which produced the highest yield of compound A_B (§ 3.5.9).

2.6.3.2.1 Purification of commercially available β-xylosidase (Sigma)

A 420 µl suspension of β-xylosidase (in ammonium sulphate) was divided into four portions. Each portion was placed in a 1.5-ml Eppendorf tube and desalted by dialysis for 3 h against 2 l of 10 mM acetic acid at pH 4.7 (adjusted with pyridine). The portions were applied to a DEAE-Sephadex A-50 column (§ 2.5.7.2.2) with 2-ml bed volume and eluted with 2 ml per step of a gradient of acetic acid: 10 mM to 1 M, Pyr^{+} salt at pH 4.7.

2.6.3.2.2 Partial purification of β-xylosidase from cucumber seeds (Mujer & Miller, 1991)

Cucumber seeds (Cucumber-Masterpiece, 20 g) were pulverised with a mixer. To this powder, 200 ml 10 mM KH_2PO_4 (pH 6.0) was added with 20 g

acid-washed sea sand and the mixture was ground in a mortar for a few minutes. Centrifugation (at 12500 rpm for 20 min and 4°C) gave a milky supernatant which was de-salted by dialysis against 1 l of extraction buffer for 24 h at 4°C with two changes. Enzyme assays were carried out in double strength McIlvaine buffer at pH 4.5 (see appendix). β-Xylosidase A activity (of Mujer & Miller, 1991) was assayed for 5 min at 65°C (100 µl enzyme solution + 100 µl 20 mM *p*-NP α- or β-D-xyloside) in a shaking water bath and stopped by immersing the tubes in ice water and by addition of 1 ml of 1 M sodium carbonate. The absorbance at 400 nm was measured immediately.

β-Xylosidase B activity was studied under similar conditions, but incubation was performed for 10 min at 50°C.

2.6.4 Elution of material from paper or staining after assay for radioactivity

A sample that had been subjected to paper chromatography and scintillation-counting and was needed for further treatment was eluted in the following way: the 1-cm paper strips were washed twice with toluene for 1 h and once for a few minutes with methylcyclohexane on a shaker. The remaining methylcyclohexane was allowed to dry overnight. The strips were then either stained (§ 2.8; if containing internal markers) or the material was eluted 5 times with water (feruloylated oligo- and polysaccharides are soluble in water) (volume depending on the size of the paper) by the method of Eshdat and Mirelman (1972) (centrifugation after each eluting step for 3 min at 2500 rpm) in a 5-ml syringe barrel. The eluates were collected and dried *in vacuo* in a Speed-Vac.

2.6.5 Purification of compounds by RPC

Disposable reverse-phase columns (size 1CC/100 mg) were pre-treated with 3 x 1 ml 100% methanol followed by 3 x 1 ml water. Aqueous solutions of feruloyl oligosaccharides were passed through a reverse-phase column (RPC) of C_{18}-substituted silica, (BondElut, Varian, Analytichem, Harbor City, California), rinsed with 3 x 1 ml of water and the retained (by hydrophobic bonding) aromatic solutes (e.g. feruloyl-[1-^{3}H]sugars) were then eluted with 50% methanol (10 ml) and dried *in vacuo*.

2.6.6 Reduction with sodium borohydride ($NaBH_4$)

Dried samples (e.g. compound A_S, § 3.4.2) were treated with sodium borohydride (0.2 ml of 0.5 M $NaBH_4$ in 1 M NH_3) for 4 h at 25°C (Fry, 1988) in a capped tube (in order to keep out carbon dioxide) (Fry, 1988) to convert the reducing termini to the corresponding alditols (e.g. A_S-ol). The time of incubation of a sample with $NaBH_4$ was 4 h in preliminary experiments, but in subsequent work this was extended to 12 h and was always repeated to be sure that the reduction was complete. The excess borohydride was destroyed by addition of 80 µl glacial acetic acid (17.4 M) and the solutions were then passed through a 1.5-ml bed volume column of Dowex-50W (§ 2.5.7.1) to remove NH_4^+ and Na^+. The samples were eluted with 8 ml water. This yielded the reduced compounds in acetic acid/boric acid, which were removed *in vacuo* and re-dried 10 times from methanol/acetic acid (10:1, v/v) in order to remove boric acid as its volatile methyl ester [$B(OMe)_3$]. According to the mechanism, 1 mol $NaBH_4$ reduces 4 mol sugar (§ 4.4.1).

2.6.7 Smith degradation

In order to investigate whether xylose was attached to O-2 or O-3 of arabinitol (see § 3.5.7.2 for the investigation of the linkage of the disaccharide within compound B and § 3.6.6 for the linkage of the additional sugar to compd B in compound C), a portion of B_S-ol was subjected to $NaIO_4$-oxidation. In preliminary studies, the dried compound was incubated with 0.1 ml of 50 mM $NaIO_4$ in 0.25 M formic acid, pH 3.7, adjusted with 1 M NaOH, for 6 days at 4°C in the dark in a capped tube. To destroy excess $NaIO_4$, 20 µl ethane-1,2-diol was added and incubated for a further 1 h (Goodman *et al.*, 1986). The products of the reaction were reduced twice with $NaBH_4$ (§ 2.6.6). After cooling to 0°C, excess $NaBH_4$ was destroyed by addition of 80 µl acetic acid, the solution was dried *in vacuo* and the residue re-dried 10 times from methanol/acetic acid (9 : 1) followed by hydrolysis with 2 M TFA for 10 min and 100°C to cleave the linkages (involving non-sugar fragments) and finally dried *in vacuo*. The products were then separated on PC in EPW_1 (§ 2.5.1) for 16 h.

The method was changed later in that, after cooling to 0°C and addition of 80 µl acetic acid, the sample was passed firstly through a 1.5-ml Dowex-

50W column (to remove sodium ions), eluted with 8 ml water, dried *in vacuo* in a Speed-Vac and re-suspended as above. Secondly it was passed through a 3-ml Dowex-1 column (to remove iodate ions), eluted with 8 ml water, dried *in vacuo* followed by severe acid hydrolysis.

2.6.8 Calculation of the specific radioactivity of [^{3}H]arabinose and [^{3}H]xylose residues in the total AIR

To calculate the specific radioactivity of [^{3}H]arabinose and [^{3}H]xylose residues in the total alcohol-insoluble residue, 110 mg AIR (for § 3.5.5.1 and 50 mg for § 3.6.2) was hydrolysed with 5 ml 2 M TFA (§ 2.6.1) for 1 h at 120°C. The supernatant was dried *in vacuo*, dissolved in 1 ml H_2O and 10 x 100 µl were subjected to PC in EPW_1 for 48 h (§ 2.5.1). A dilution series of xylose and arabinose was used as external markers (in a logarithmic scale of 2 µg, 5 µg, 10 µg, 20 µg .... 320 µg per loading).

One of the ten tracks was stained (§ 2.8.1) and was used for estimating the amount of arabinose that had been released after severe acid hydrolysis. From 11 mg fescue cell walls approximately 1 mg arabinose and approximately 0.2 mg xylose was observed (by visual comparison with the external marker series). From the nine other tracks, 7-cm parts were cut out:

(a) blank: -7 cm to origin
(b) arabinose: 28 to 35 cm
(c) xylose: 46.6 to 53.6 cm

The sugars (including blank) from each 7-cm strip were eluted with water, dried and each re-dissolved in 5 ml water. Portions from each 5 ml were assayed ① for radioactivity (§ 2.7.1.2) (6 x 33 µl) and ② for reducing sugars by the PAHBAH-assay (§ 2.7.2) (10 x 33 µl aliquots of a 10-fold dilution), using L-arabinose and D-xylose as standards.

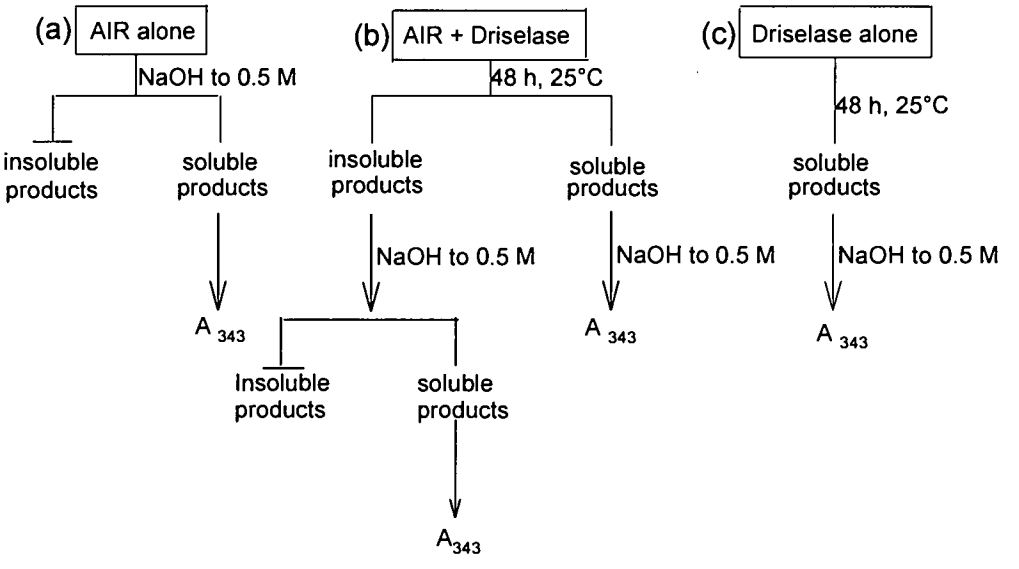
For the standard curves, L-arabinose and D-xylose were previously dried in a thermostat vacuum oven over phosphorus pentoxide for 9 h. A dilution series was prepared for each sugar and the absorbance measured at 410 nm.

2.6.9 Hydrolysis of non-labelled AIR

2.6.9.1 AIR for treatment with Driselase (§ 3.10.1)

A suspension culture of *Festuca arundinacea* (250 ml, 4 days after sub-culture) or *Zea mays* was passed through a Büchner funnel and the cells were washed for 48 h (with several changes) with 2 l of 80% ethanol and afterwards allowed to dry.

To calculate the percentage of soluble products that could be released from fescue and maize cell walls by Driselase (§ 3.10.1), three preparations were set up: (a) cell walls alone, (b) Driselase incubated with cell walls in buffer and (c) Driselase incubated alone in buffer. The following diagram shows the general procedure:



For (a), a portion (100 mg) of fescue AIR was incubated with 5 ml 0.5 M NaOH for 1 h. After centrifugation, the A_{343} of the supernatant was read. For (b), 200 mg was hydrolysed with 50 mg Driselase in 10 ml buffer (§ 2.6.3.1.2). After centrifugation and washing of the pellet, the supernatants were combined (11 ml) and 0.5 ml (= 4.55%) was incubated with 0.5 ml 1 M NaOH. After 1 h, the absorbance at 343 nm was measured. The insoluble residue was re-suspended in 10 ml 0.5 M NaOH, incubated at 25°C for 1 h, and centrifuged; the A_{343} of the supernatant was then read. For (c), 25 mg Driselase was incubated with 5 ml buffer for 48 h, 0.5 ml was mixed with 0.5 ml 1 M NaOH. After 1 h, the A_{343} of the supernatant was then read.

Maize AIR (100 mg in 5 ml buffer) was treated as described above under fescue hydrolysis. Here 0.5 ml (out of 6 ml; = 8.33%) of the solubilised products was mixed with 0.5 ml 1 M NaOH for 1 h.

For the calculation of the percentage of the soluble products which were oligomeric, a portion (2 ml = 36.4 mg fescue AIR and 9.1 mg Driselase) of the soluble products released by Driselase was mixed with 500 µl formic acid and subjected to PC (as a 37-cm streak) in BAW (§ 2.5.1). 1-cm Strips of the paper were treated with 5 ml 0.5 M NaOH for 1 h followed by measurement of the absorbance at 343 nm. A Driselase-alone sample (25 mg Driselase) incubated in 5 ml buffer for 48 h served as a control. Of this solution, 1.818 ml (= 9.1 mg Driselase) was mixed with 455 µl formic acid and treated as above. For the calculation of the percentage of the Driselase-solubilised products of maize AIR, 2.184 ml (= 36.4 AIR and 9.1 mg Driselase) was taken as described above.

2.6.9.2 AIR from leaves (for § 3.12)

All material was either collected in Spain, Edinburgh or harvested in the greenhouse at Edinburgh University. Routinely, 5 g leaves were crushed with a pestle and mortar in liquid nitrogen. Sea sand (2 g) was added and homogenised with 10 ml 80% EtOH for 5 min. The supernatant was rejected. After two repetitions the AIR was allowed to dry for 48 hours.

A portion of the dried AIR (100 mg from each species) was hydrolysed with 5 ml 0.1 M TFA (§ 2.6.1) and the hydrolysate subjected to PC in BAW (§ 2.5.1). From the hydrolysates of 14 different species (§ 3.12.2.1), compounds A and B were eluted with water (§ 2.6.4), enriched in phenolic-containing material by reverse-phase column chromatography (§ 2.6.5) and further purified by PC in BEW (§ 2.5.1). The pre-purified compounds were applied on to an analytical reversed-phase HPLC column (§§ 2.5.3 and 3.12.2.2) and their ratio calculated. For this, compound A from each species and compound B from three species (*Phleum*, *Anthoxanthum* and *Festuca arundinacea*) were quantified *in silico* by the integrator and in addition, compound B from all species by triangulation. For the three species mentioned the area found by integration was divided by the area found by triangulation. The average of the results was calculated and then the area found by integration of compound A (of all species) was divided by this average.

2.6.10 Preparation of radiolabelled AIR

A suspension culture of *Festuca arundinacea* (§ 2.6.9.1) was incubated under standard conditions with 47 MBq of L-[1-^{3}H]arabinose for 5 h. The cells were then washed and allowed to dry as described in § 2.6.9.1.

^{14}C-labelled AIR (labelled in the ferulate moieties) was obtained by feeding 2 MBq [^{14}C]cinnamate to a suspension culture of fescue (4 days after sub-culture) for 7 days under sterile conditions. The cells were then washed and allowed to dry as described above.

To investigate the *in vivo* feruloylation of oligosaccharide moieties of arabinoxylan (§ 3.9), a suspension culture of fescue (4 days after sub-culture) was incubated with [^{14}C]ferulate and [^{3}H]arabinose. For this, 60 ml of suspension culture was transferred into a 250-ml flask and left shaking for 2 hours. At time zero (t_0) 480 MBq L-[1-^{3}H]arabinose (in 3 ml water) and 36 MBq [^{14}C]ferulate (prepared and purified from [*methyl*-^{14}C]ferulic acid (in 250 µl DMSO) were added. At time intervals, 2-ml samples of the cell suspension were removed into 8 ml EtOH, passed through Poly-Prep columns (Bio-Rad) and washed overnight with 200 ml 80% EtOH followed by 20 ml acetone. After drying the weight of the AIR (at each time-point) was measured.

To identify the additional ester group in compounds D and E (§§ 3.7.5 and 3.8.5), suspension culture of fescue was fed with Na[^{14}C]acetate over a time course (designed to monitor the highest yield of incorporation products) from 0 h, 0.5 h, 1 h, 2 h ... to 72 h. To 50 ml suspension culture, 2 MBq Na[^{14}C]acetate was added and 5-ml samples were taken out into 1 ml EtOH. It turned out that 32 h gives the highest yield of ^{14}C-AIR. Consequently, a further portion of suspension culture cells was fed with Na[^{14}C]acetate (0.5 µCi) over 32 hours.

2.6.11 Methylation analysis

A portion (0.6 mg) of HPLC-purified compound B was methylated by the Hakomori method: treatment of DMSO with sodium hydroxide generates a methylsulphinyl carbanion to catalyse the ionisation of the hydroxyl groups followed by methylation with methyl iodide. The permethylated glucoside is hydrolysed in a formic acid-TFA mixture, methylated derivatives of L-arabinose and D-xylose were reduced with $NaBD_4$, peracetylated to the

corresponding alditol acetates and analysed by GC-MS, which gave two major peaks. Each peak was further analysed by MS. The analyses were performed by Dr V. Farkaš in the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia.

2.7 Assays

2.7.1 Radioactivity

2.7.1.1 Non-aqueous samples

Non-aqueous samples (e.g. strips of chromatography paper) were analysed by using non-Triton scintillation cocktail (NTS) containing 0.5% (w/v) PPO (2,5-diphenyloxazole) and 0.05% (w/v) POPOP (1,4-bis-(5-phenyloxazole-2-yl)benzene) in toluene (Fry, 1988). Routinely, 2 ml of this cocktail was added to wet a strip of 1 cm x 4 cm of Whatman 3MM paper. The counting efficiency for ^{3}H-labelled sugars was approximately 7%.

2.7.1.2 Aqueous samples

Aqueous counting was mostly used in order to increase the counting efficiency for ^{3}H (about 6 times compared with NTS). For this, samples (1- cm paper strips) were washed (after being used for NTS) twice with toluene for 1 h and once with methylcyclohexane for 15 min and then dried overnight. The strips were incubated (within the tubes) with 1 ml water for 30 min and then mixed with ten volumes of Triton scintillant made up of toluene/Triton X-100 (2:1, w/v) containing 0.33% (w/v) PPO and 0.033% (w/v) POPOP (Fry, 1988).

2.7.2 Reducing sugars (PAHBAH)

p-Hydroxybenzoic acid hydrazide (10 ml of 5% solution) in 0.5 M HCl was freshly mixed with 40 ml 0.5 M NaOH. A portion (750 µl) of this solution was added to 250 µl aqueous samples, incubated in a boiling water bath for 5 min and cooled and the absorption measured at 410 nm (after Lever, 1972). L-Arabinose and D-xylose were used as standards.

2.7.3 Hexoses (anthrone)

To 0.5 ml aqueous solution (100 µl sample + 400 µl water) was added 1 ml of 0.2% anthrone in concentrated sulphuric acid. The solution was mixed carefully and incubated in a boiling water bath for 5 min. After cooling, the absorbance was measured at 620 nm. The method was used to detect for free and polymer-bound hexoses (after Dische, 1962). Hexoses give blue and most other sugars greenish colours.

2.7.4 Xylosidase assays

2.7.4.1 Methyl α- and β-xylosidase

Methyl α- and β-D-xylosides (each 100 µl of 10 mM, repeated with 20 mM) were incubated with 100 µl enzyme solution (β-xylosidase) (after Fry, 1988) for 4 h at 25°C. The hydrolysis was stopped with 25 µl formic acid and the products were diluted with 150 µl water (to give 250 µl final sugar solution) and mixed with PAHBAH solution (§ 2.7.2). After incubation in a boiling water bath for 5 min and cooling, the absorbance at 410 nm was read as a measure of free xylose formed.

2.7.4.2 [^{3}H]Isoprimeverase

A portion of [^{3}H]isoprimeverose (5 µl; for preparation see § 2.3.2.2) was treated in the same way as methyl α- and β-xylosides, then subjected to PC in BAW (§ 2.5.1) and assayed for [^{3}H]xylose in NTS (§ 2.7.1.1) (after Fry, 1988). Isoprimeverose is a disaccharide containing an α-xylose residue. Thus, the β-xylosidase (after purification, § 2.6.3.2) can be tested for α-xylosidase contamination.

2.7.4.3 *p*-Nitrophenyl xylosidase

p-NP α/β-xyloside	+	H_2O	→	Xylose	+	*p*-Nitrophenol
(colourless)						(yellow in alkali)

p-Nitrophenyl α-xyloside and *p*-nitrophenyl β-xyloside (each 0.5 ml of 5 mM) in 50 mM acetic acid, pH 4.7 (adjusted with 1 M NaOH) were incubated

with 0.5 ml enzyme solution (after Pierrot & Wielink, 1977) for 4 h and absorbance measured as described in § 2.6.3.2.2.

2.8 Staining methods

To visualise the internal and external markers, applied to both paper electrophoretograms and paper chromatograms, one of the following techniques were used.

2.8.1 Aniline hydrogen phthalate

The stock solution was 1.6% phthalic acid dissolved in acetone/diethyl ether/water (49:49:1,v/v/v). Immediately before use 0.5% (v/v) aniline was added. To stain monosaccharides and reducing disaccharides papers were then dipped through this solution and dried for a few minutes in the fume cupboard. After heating for 5 min at 105°C the different colours could be seen: pentoses - red, hexoses - brown and uronic acids - orange (Partridge, 1949; modified by Fry, 1988).

2.8.2 Alkaline silver nitrate

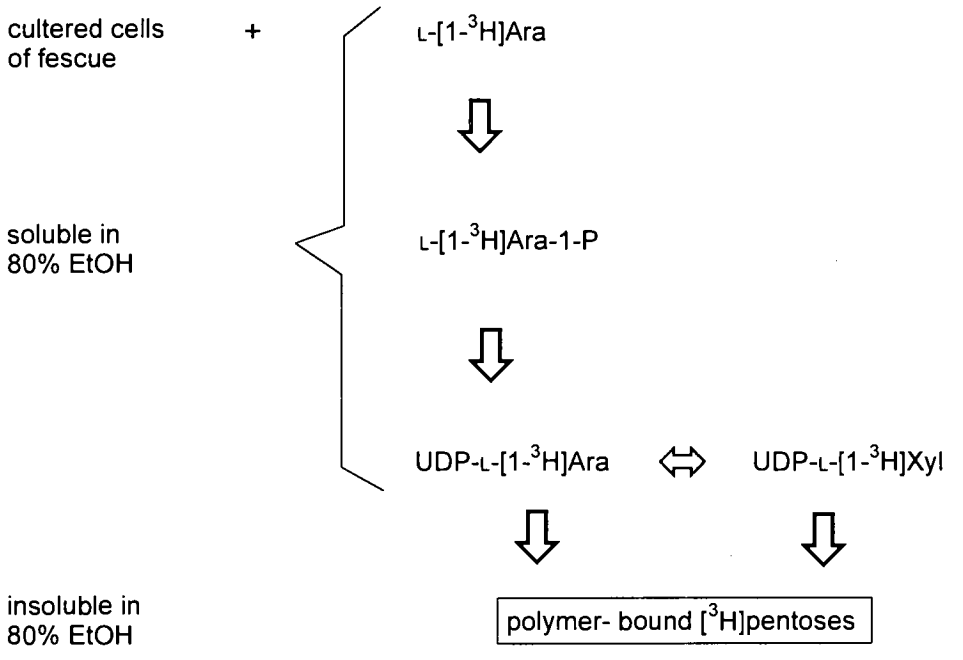
An alkaline silver nitrate reagent stains monosaccharides, oligosaccharides and alditols. Papers were dipped through a solution of silver nitrate in acetone (0.5 ml saturated aqueous $AgNO_3$/100 ml acetone and water to re-dissolve), allowed to dry and then re-dipped through an ethanolic sodium hydroxide solution (1.25 ml 10 M NaOH/100 ml absolute ethanol). In order to store the chromatograms they were finally dipped in 10% sodium thiosulphate solution (otherwise they turn black) (Trevelyan *et al.*, 1950).

For chromatograms run in solvents containing borate, the method was slightly changed. Instead of ethanolic sodium hydroxide, a spray of 2% NaOH and 4% pentaerythritol in 80% ethanol was used. For preparation of this reagent, 2% NaOH was dissolved in 80% ethanol first and then 4% pentaerythritol added.

3. Results

3.1 Feruloylated oligosaccharides released from AIR by mild acid

A suspension culture of *Festuca arundinacea* was incubated under standard conditions with L-[1-^{3}H]arabinose (§ 2.6.10) to obtain polymer-bound [^{3}H]pentoses:



The cells were then washed with 80% ethanol to yield an alcohol-insoluble residue (AIR), which was allowed to dry. The approximate specific activity of the ^{3}H-AIR was 0.79 MBq/mg dry weight. Complete acid hydrolysis gave [^{3}H]Ara and [^{3}H]Xyl (§ 2.6.8), which, in view of the well-established pathway (above), are assumed to be L- and D-, respectively.

One portion of the ^{3}H-AIR was subjected to mild acid (§ 2.6.1) to hydrolyse a proportion of the glycosidic bonds. The hydrolysate was subjected to preparative PC in BAW (§ 2.5.1) (Fig. 3.1). Eight blue fluorescent zones (A to H; H = origin), turning to intense blue-green under ammonia vapour, were located. The fluorescence characteristics strongly suggested feruloyl derivatives.

Since compounds A to H can be released by mild acid hydrolysis (§ 2.6.1) it is very likely that the reducing terminal sugar of each compound had been linked by its furanose form within its parent polymer (see introduction).

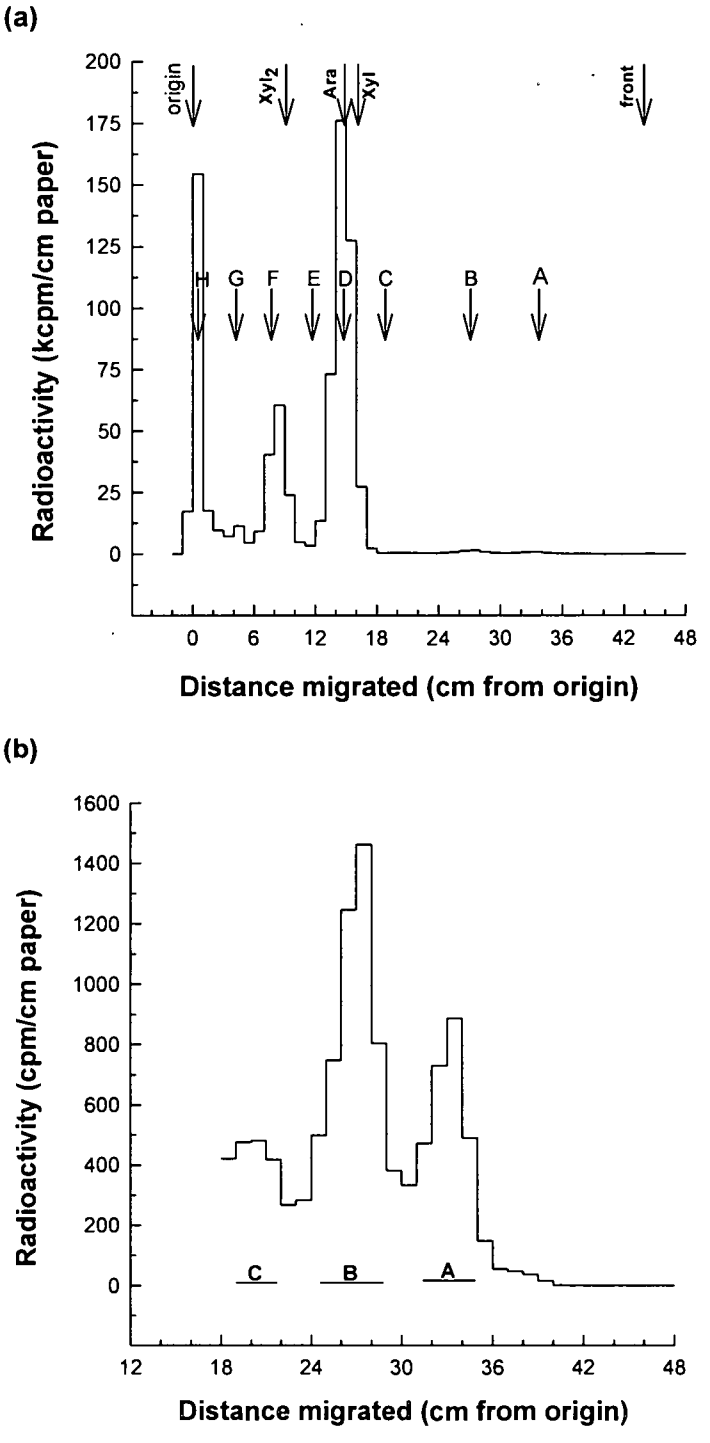


Fig. 3.1: PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of (*pentosyl*-^{3}H)-labelled fescue AIR.

(a) Eight blue fluorescent zones (A to H); Xyl_2, Ara and Xyl = marker sugars; front = solvent front. The arrows indicate the middle of each zone.

(b) A section of (a) to clarify the radioactivity present in the fluorescent products A, B and C (18 to 48 cm).

3.1.1 Purification of compounds A to H

The fluorescent zones (A to H) were eluted with water (§ 2.6.4), enriched in phenolic-containing material by reverse-phase column chromatography (§ 2.6.5) and further purified by PC in BEW (§ 2.5.1). Each compound re-ran as a single peak (Fig. 3.2 shows this for compounds A, B and C). Compounds of interest (A, B, C, D and E) were freed of scintillant (§ 2.6.4) and re-eluted with water. Additional purification could be achieved by PE in borate-buffer (§ 2.5.2). Figure 3.3 indicates this for compounds A and B.

3.1.2 Alkaline hydrolysis

The compounds were subjected to alkaline hydrolysis to yield the de-feruloylated ^{3}H-labelled sugar moieties (A_S to H_S; see also §§ 3.6.7.1, 3.7.4.1 and 3.8.4.1). Each compound gave a single radioactive product with the exception of compound C which gave two: C_S and C_S' (§ 3.6.7.1, Fig. 3.47). Table 3.1 summarises the chromatographic data for compounds A to E and A_S to E_S.

compound	R_{Ara} (in BAW)	R_f (in BEW)	R_{Ara} (in EPW)	R_{Ara} (in BPW)	m_{Ara} (borate-buffer)
A	2.21	0.74	nd	nd	0.46
A_S	1.00	nd	1.00	1.00	1.00
B	1.86	0.57	nd	nd	0.69
B_S	0.64	nd	0.27	nd	nd
C	1.36	0.38	nd	nd	nd
C_S	0.50	nd	0.11	nd	nd
C_S'	0.70	nd	0.34	nd	nd
D	0.95	0.33	nd	nd	nd
D_S	0.38	0.15	nd	0.81	nd
E	0.73	0.29	nd	nd	nd
E_S	0.25	0.09	nd	0.74	nd

Table 3.1: Chromatographic data obtained for compounds A to E and A_S to E_S.

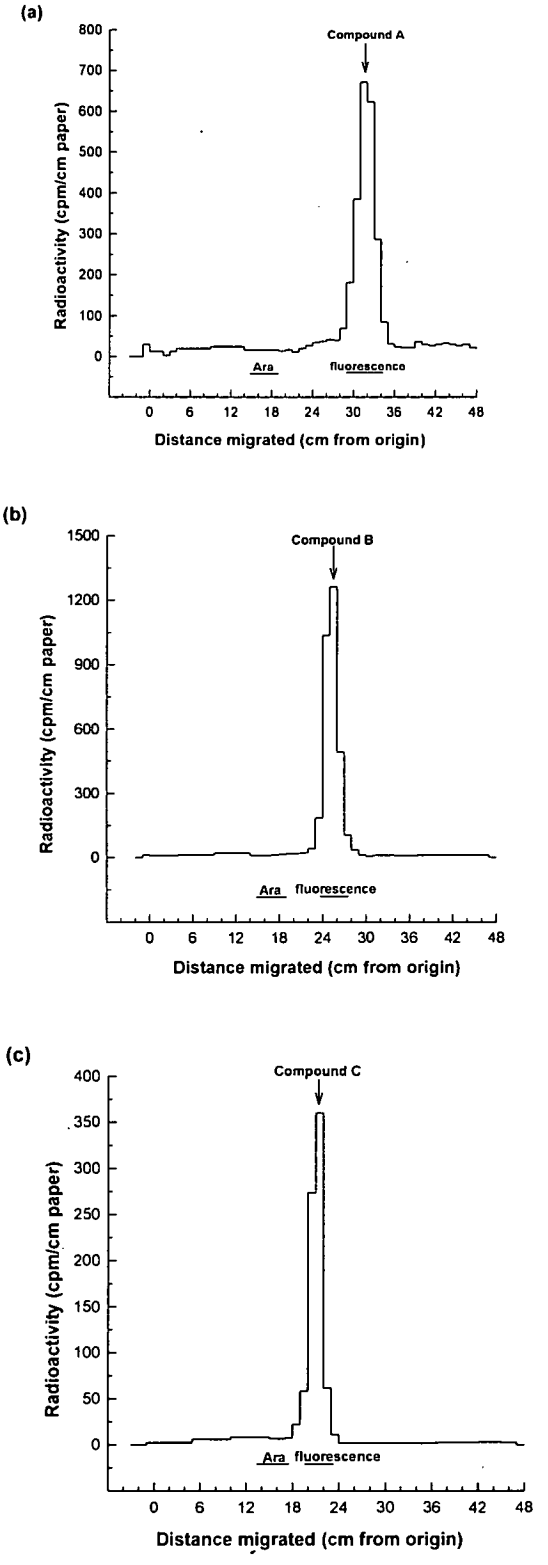


Fig. 3.2: PC in BEW (§ 2.5.1) of ^{3}H-labelled compounds A (a), B (b) and C (c) from figure 3.1 after further purification by reverse-phase column chromatography. Ara was used as an external marker. "Compd A", "Compd B" and "Compd C" show their approximate position (Table 3.1).

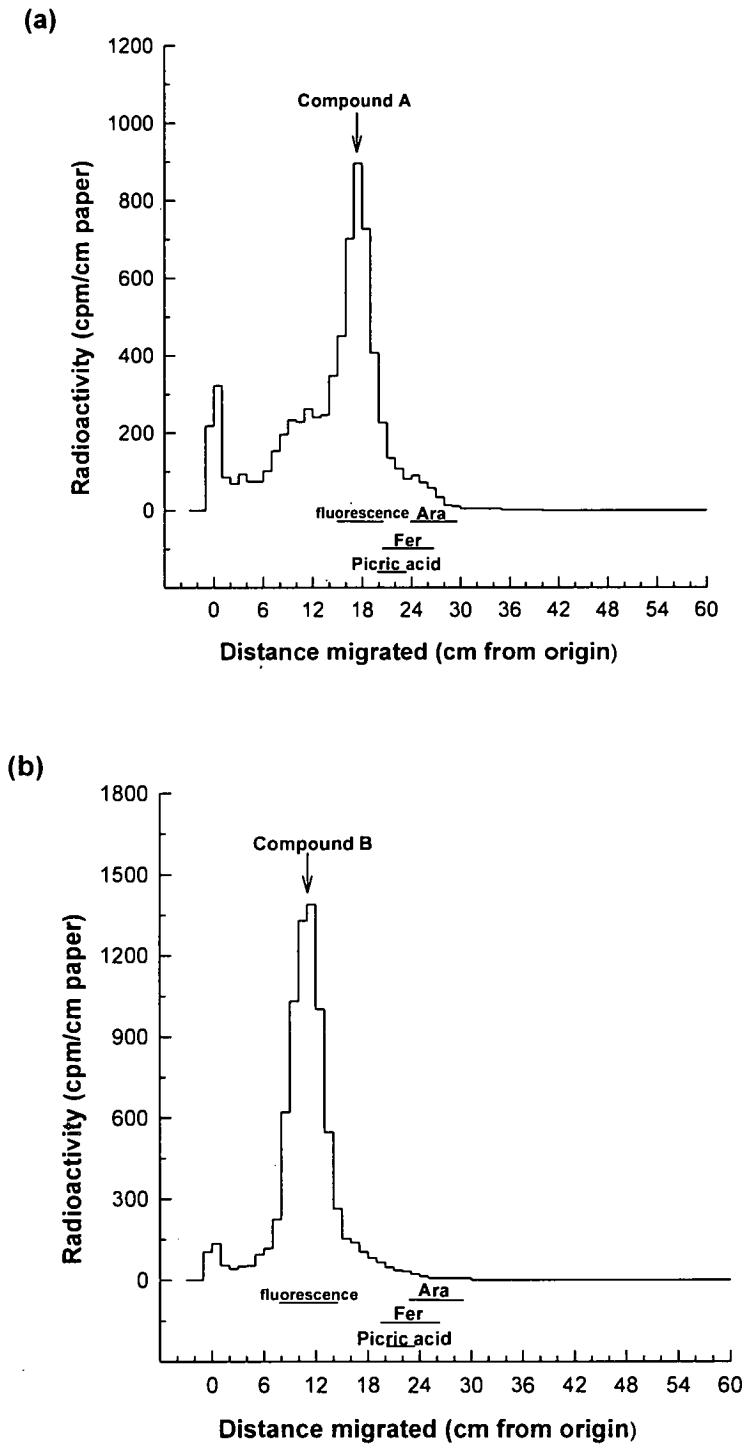


Fig. 3.3: PE in borate buffer (§ 2.5.1) of ^{3}H-labelled compounds A (a) and B (b). Ara, Fer and picric acid were used as external markers. "Compd A" and "Compd B" show their approximate position (Table 3.1).

3.2 Working hypothesis for the structure of a feruloylated arabinoxylan

Further work was carried out to examine in detail the structure of compounds A, B, C, D and E. Figure 3.4 shows a possible arabinoxylan composition of fescue and the route of its hydrolysis. §§ 3.3 to 3.10 describe work designed to test this working hypothesis.

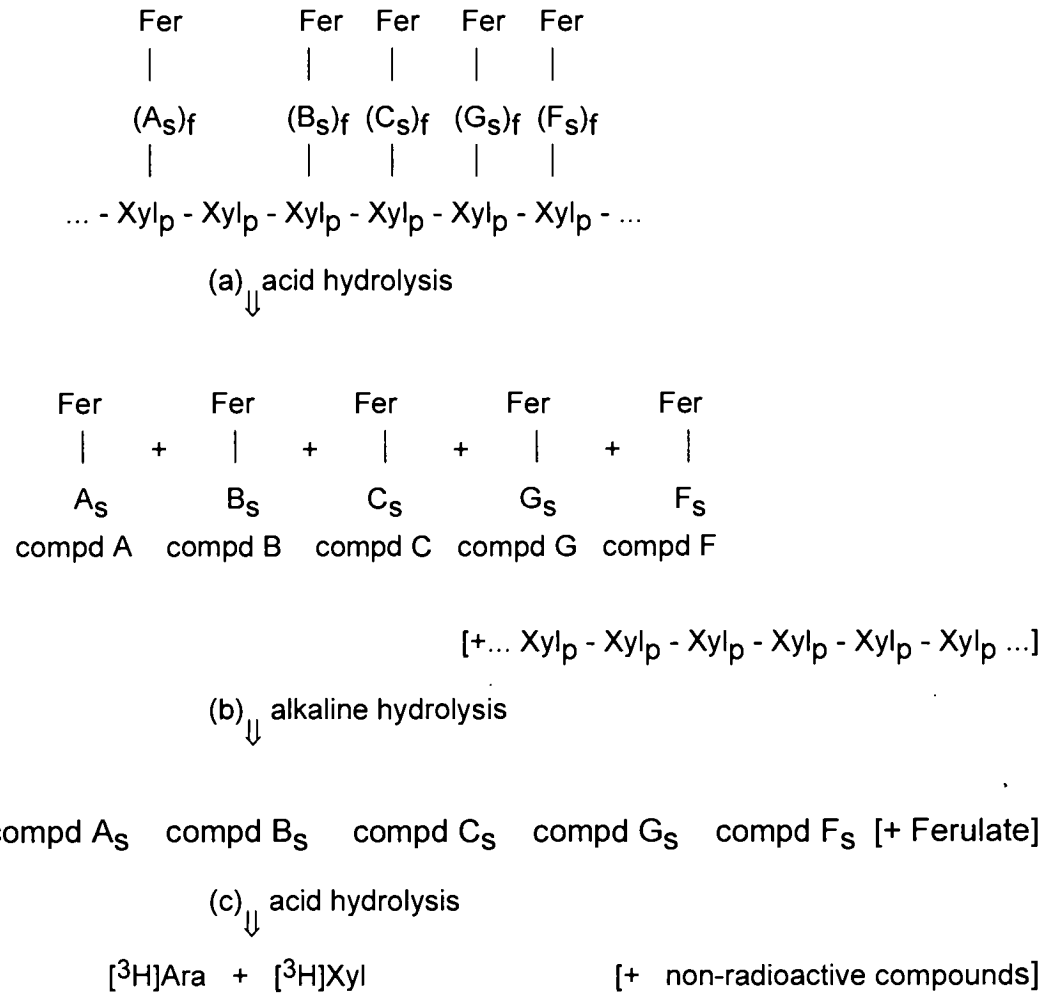


Fig. 3.4: An assumed structure of arabinoxylan of fescue

... - Xyl_p - Xyl_p - Xyl_p-... = xylan-backbone; $_p$ = pyranose-form; $_f$ = furanose-form; Fer = ferulic acid (esterified).

(a) Cleavage of products (e.g. compounds A, B, C, G and F) form the xylan backbone (see introduction) by mild acid hydrolysis (§ 2.6.1).

(b) De-feruloylation of compounds by alkaline hydrolysis (§ 2.6.2).

(c) Severe acid hydrolysis (§ 2.6.1) resulting in the release of monosaccharides.

3.3 *O*-Feruloylated products

To demonstrate the feruloyl ester bond within compounds A and B, alkaline hydrolysis was conducted (§ 2.6.2) and the UV absorption spectrum measured. Figure 3.5 shows the comparison of pure compound A (in H_2O); compound A immediately after addition of sodium hydroxide; and the products after 60 min hydrolysis. Figure 3.6 shows similar data for compound B.

In both compounds a clear shift of the absorption maximum from 323 nm to 373 nm and finally to 343 nm can be seen. Very similar spectra have been published (Smith & Hartley, 1983), indicating an *O*-feruloyl ester bond.

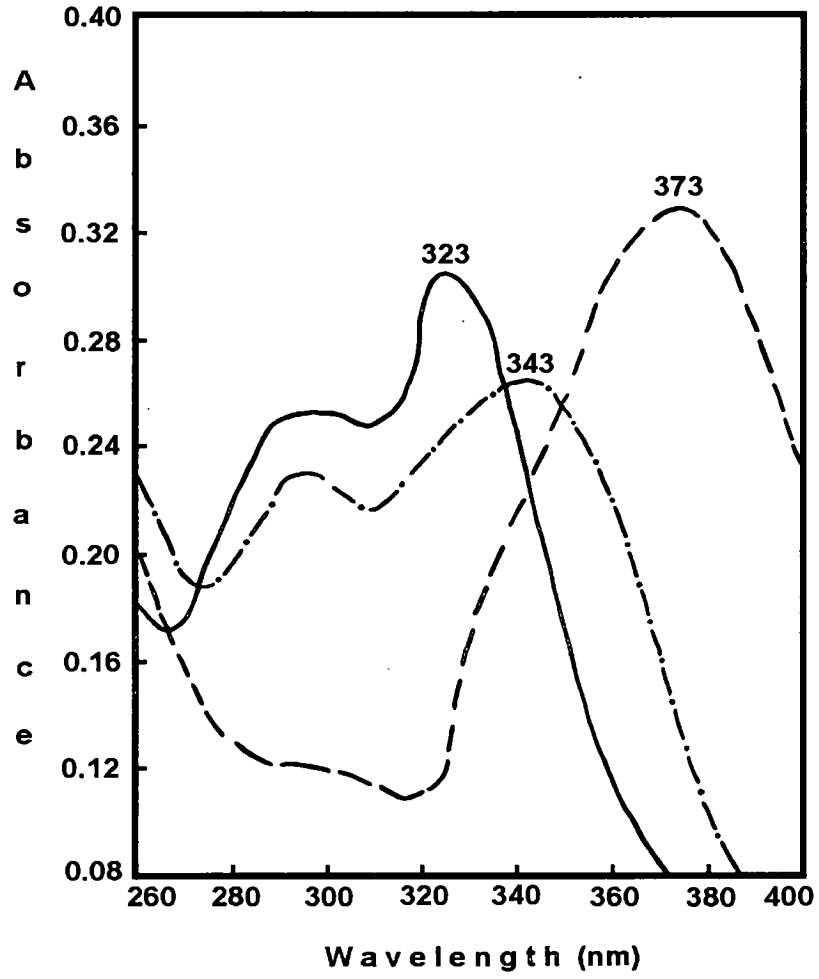


Fig. 3.5: UV absorption spectrum of compound A, — pure compound A (in H_2O); --- after 30 s and –·– after 60 min alkaline hydrolysis (§ 2.6.2).

The initial shift from 323 nm to 373 nm is due to the formation of the sodium phenolate salt whereas the shift from 373 nm to 343 nm represents the formation of sodium trans-ferulate (Smith Hartley, 1983).

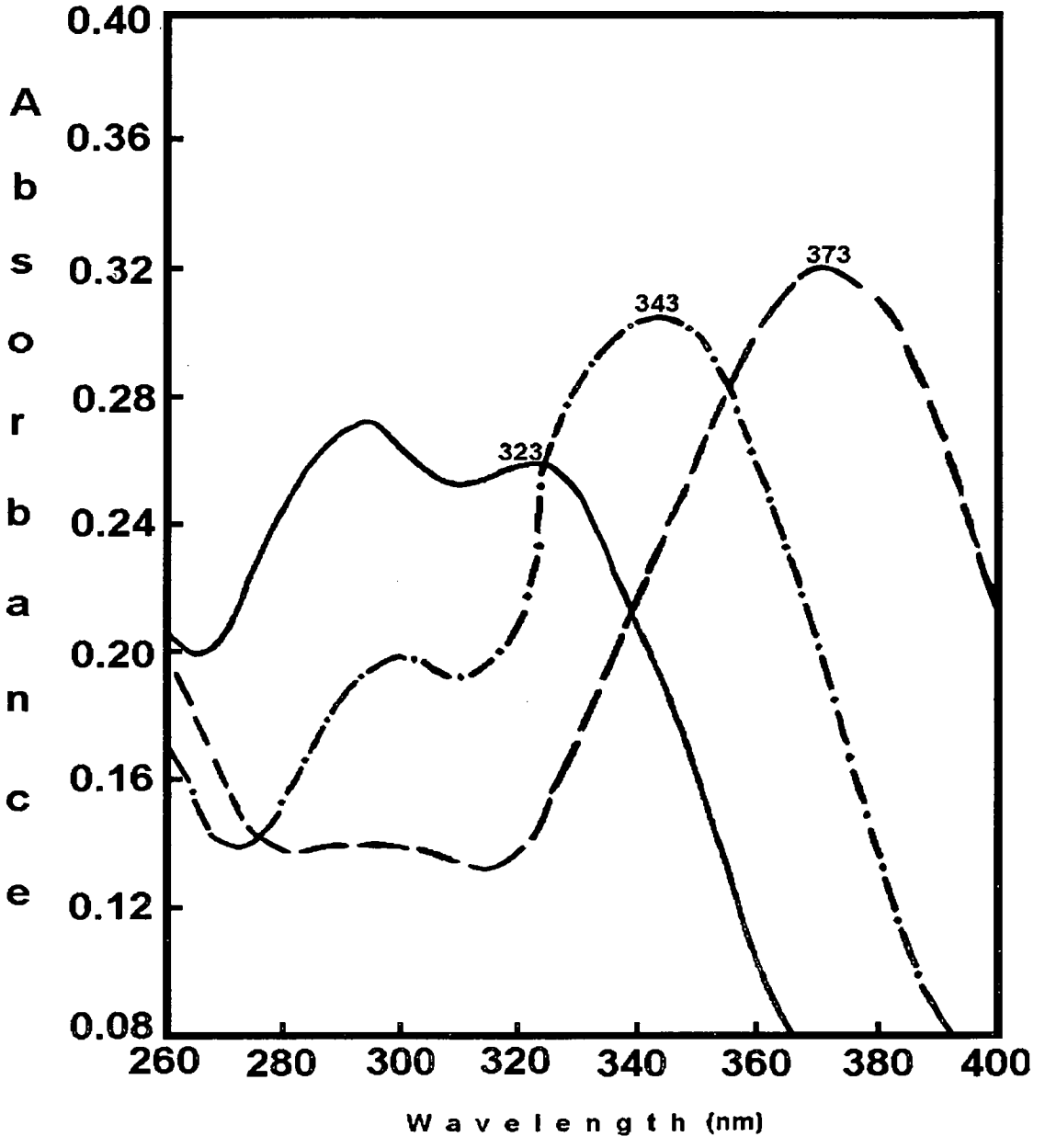


Fig. 3.6: UV absorption spectrum of compound B, — pure compound B (in H_2O); --- after 30 s and –·– after 78 min alkaline hydrolysis (§ 2.6.2).

3.4 Investigation of compound A

3.4.1 Alkaline hydrolysis with internal marker

Alkaline hydrolysis of ^{3}H-labelled compound A yielded a single radioactive product (compd A_S) (Table 3.1) which co-chromatographed with authentic non-radioactive arabinose (Fig. 3.7). In order to demonstrate this, 1-cm strips containing the internal marker arabinose (and 3 adjacent strips) were stained after scintillation counting (§§ 2.6.4 and 2.8.1).

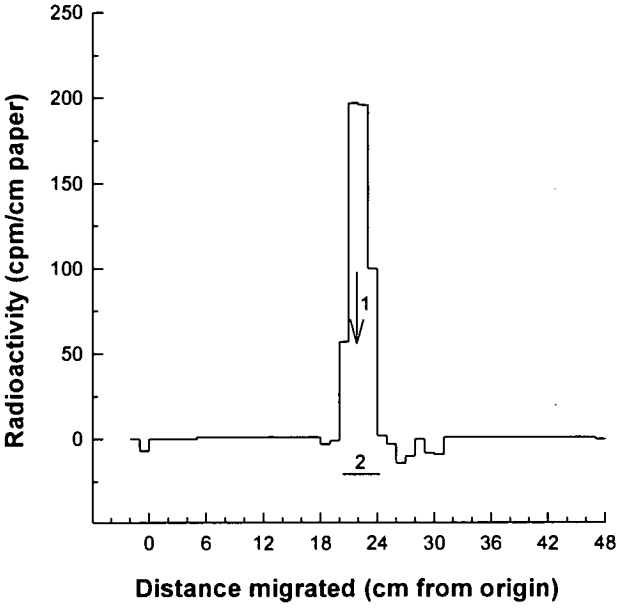


Fig. 3.7: PC in EPW_1 (§ 2.5.1) of ^{3}H-labelled compound A_S. Arabinose was used as a marker; 1: arabinose (external), 2: arabinose (internal).

3.4.2 Reduction of compound A

If compound A is composed solely of arabinose and ferulic acid, that after reduction of compound A_S with sodium borohydride (§ 2.6.6) it can be expected that [^{3}H]arabinitol would be obtained.

Indeed, treatment of compound A_S with sodium borohydride yielded only [^{3}H]arabinitol (data not shown). It can be concluded that compound A was an *O*-feruloyl-L-arabinose, possibly 5-*O*-feruloyl-L-arabinose, which is widespread in graminaceous cell walls (Gubler, *et al*, 1985; Kato and Nevins, 1985; Ahluwalia and Fry, 1986; Mueller-Harvey et al., 1986).

3.4.3 Kinetics of alkaline hydrolysis

3.4.3.1 Determination of the half-life

To examine the stability of the ester bond within compound A, alkaline hydrolysis (§ 2.6.2) was conducted at pH ~13.3 and 25°C and monitored chromatographically. Figure 3.8 shows chromatograms of the material after 0 min and 7.5 min. Complete breakdown took place within the first 7.5 min (Fig. 3.9).

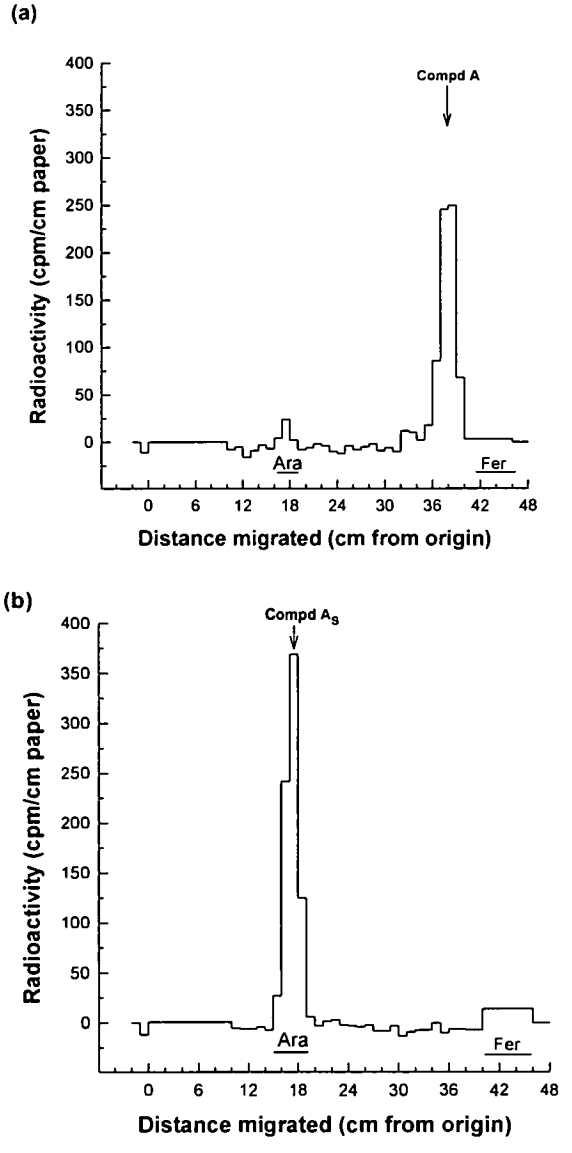


Fig. 3.8: PC in BAW (§ 2.5.1) of product of Compound A after alkaline hydrolysis (§ 2.6.2) for (a) 0 min and (b) 7.5 min. Ara and Fer served as external markers. "Compd A" and "Compd A_S" show the approximate position of these compounds (Table 3.1).

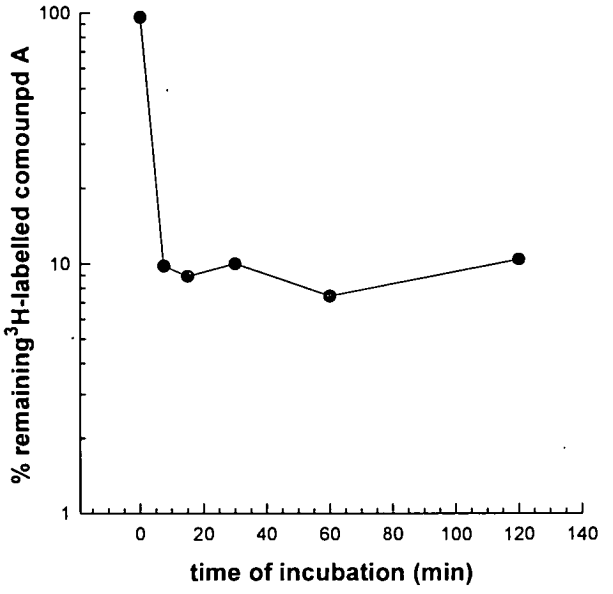


Fig. 3.9: Alkaline hydrolysis of ^{3}H-labelled compound A (§ 2.6.2) over a time course.

In order to determine the half-life of compound A, alkaline hydrolysis of non-radioactive compound A at pH ~12.8 and 25°C was performed over a shortened time course with the absorbance read at 380 nm (Fig. 3.10). The half-life was ~2 min.

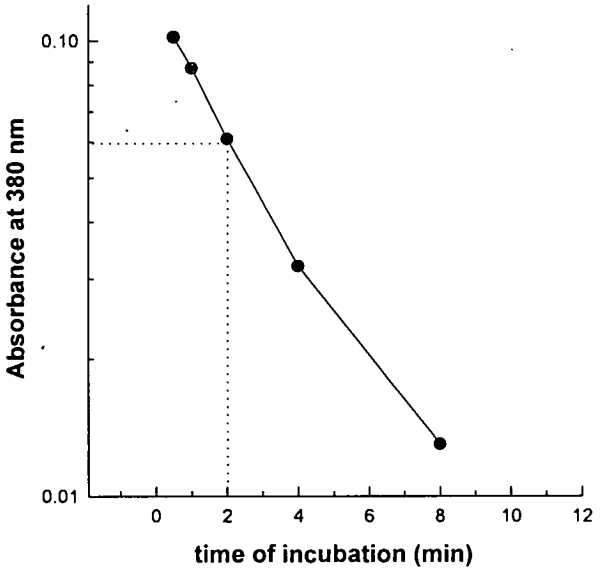


Fig. 3.10: Short-term kinetics of alkaline hydrolysis of compound A (§ 2.6.2). A_{380} indicates remaining compound A.

3.4.3.2 Test for multiple esters

It cannot be assumed that ferulate is the sole ester-linked group present in compound A. To test for multiple esters in compound A, partial saponification was carried out. If compound A, for instance, is *O*-feruloyl arabinose, we would expect to get a single radioactive product even after partial alkaline hydrolysis. If there were another ester linkage e.g. of an *O*-acetyl group, we would see at least two radioactive products (Fig. 11).

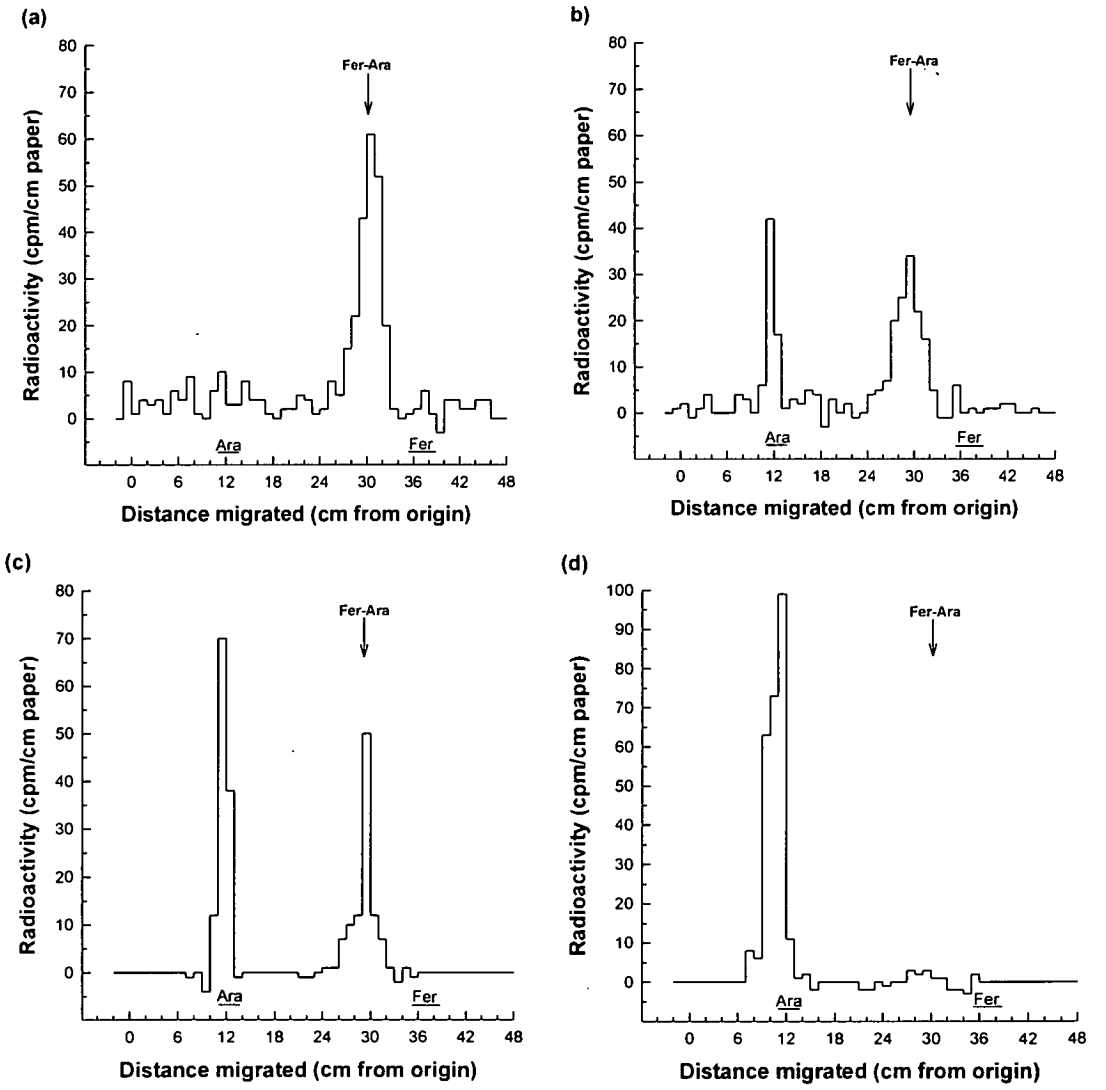


Fig. 3.11: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.6.2) products of ^{3}H-labelled compound A formed after (a) 0 min, (b) 0.5 min, (c) 1 min and (d) 2 min. Ara and Fer served as external markers. "Fer-Ara" (Compd A) shows its approximate position (Table 3.1).

Figure 3.11 reveals that no intermediate product was formed. One may conclude that ferulate is the sole ester-linked group in compound A.

3.4.4 Position of the ester-linkage

To test whether the feruloyl group of compound A is indeed linked to *O*-5 of arabinose, the ^{3}H-labelled compound was subjected to PC in BAW (§ 2.5.1) with authentic 5-*O*-feruloyl-L-arabinose (from maize) as an internal marker. Figure 3.12 demonstrates that the ^{3}H profile of compound A is consistent with the internal standard 5-*O*-feruloyl-L-arabinose.

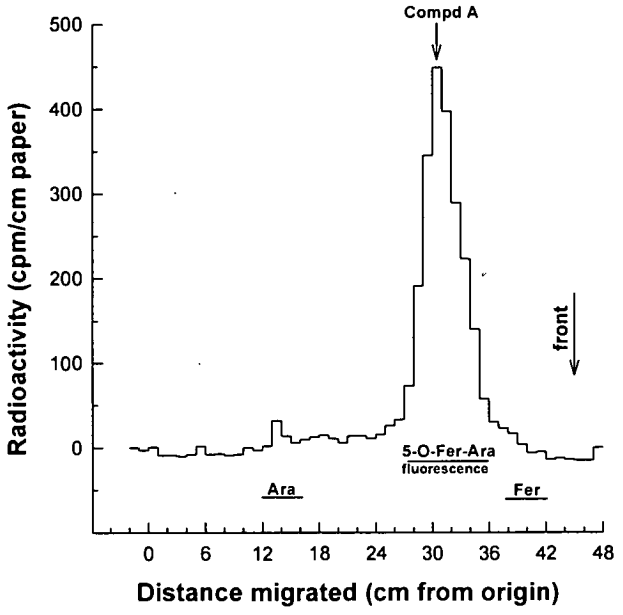


Fig. 3.12: Co-chromatography by PC in BAW (§ 2.5.1) of ^{3}H-labelled compound A with internal marker authentic 5-*O*-feruloyl-L-arabinose (of maize). Ara and Fer were used as external markers. "Compd A" shows its approximate position (Table 3.1).

Taken together, the evidence shows that arabinose is the sugar moiety within compound A and that ferulic acid is esterified (as the sole ester present) to *O*-5 of arabinose.

3.5 Investigation of compound B

3.5.1 The proposed structure of compound B

The supposed structure of compound B and its linkage to the backbbone are given in Figure 3.13. The following sections will provide evidence for this structure.

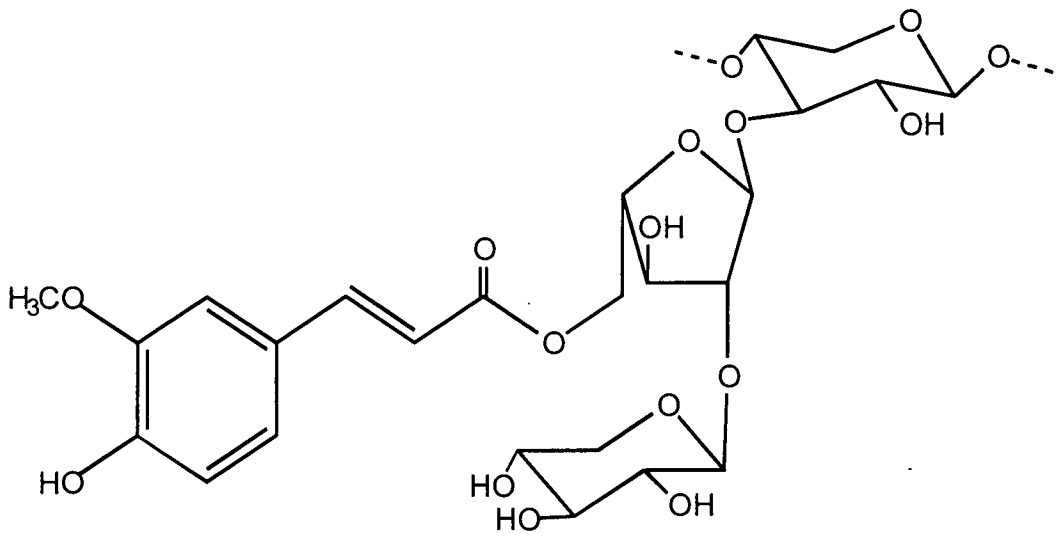


Fig. 3.13: The proposed structure of compound B connected to its parent polymer.

3.5.2 Alkaline hydrolysis with internal marker

Alkaline hydrolysis of compound B yielded a single radioactive product (compound B_S). B_S migrated significantly slower than compound A_S, indicating that it was an oligosaccharide (Fig. 3.14).

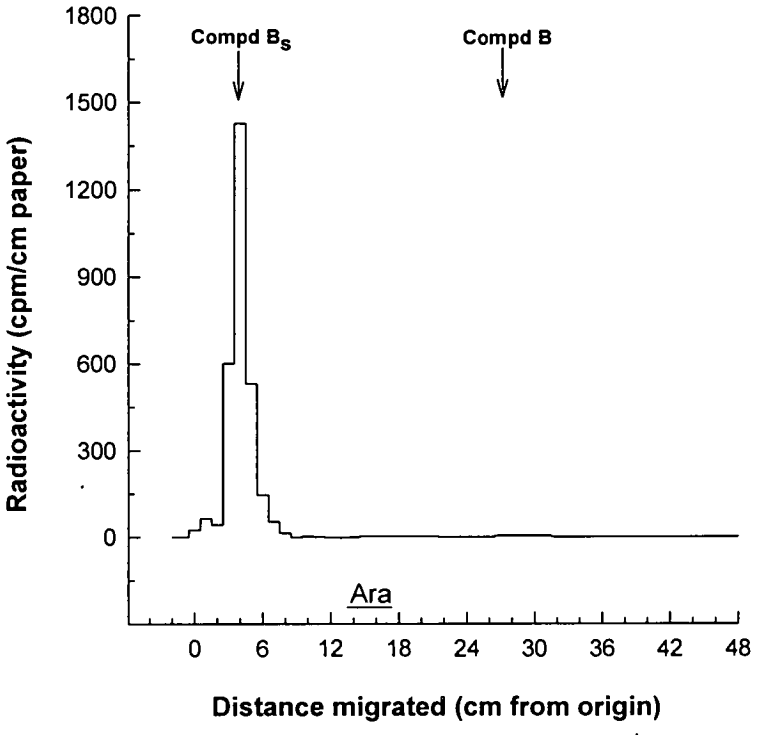


Fig. 3.14: PC in EPW_2 (§ 2.5.1) of ^{3}H-labelled compound B_S obtained from compound B by alkaline hydrolysis (§ 2.5.2). Ara was used as external an marker. Compounds B and B_S indicate their approximate position (Table 3.1).

3.5.3 Kinetics of alkaline hydrolysis

3.5.3.1 Determination of the half-life

To examine the alkali lability of the *O*-feruloyl ester bond within compound B, alkaline hydrolysis of ^{3}H-labelled compound B was conducted at pH ~13.3 and 25°C (§ 2.6.2) and the products were examined chromatographically. The half-life was estimated at ~10 min (Fig. 3.15).

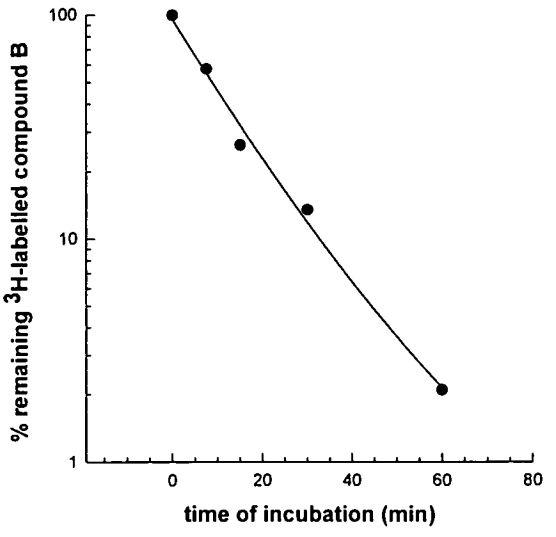


Fig. 3.15: Alkaline hydrolysis (§ 2.6.2) of ^{3}H-labelled compound B over a time course.

In order to determine the half-life of compound B more accurately, alkaline hydrolysis of non-radioactive compound B at pH ~12.8 and 25°C was performed (§ 2.6.2) over a shortened time course and the absorbance read at 380 nm (Fig. 3.16). The half-life was 14 min. Thus, compound B was appreciably more alkali-stable than compound A.

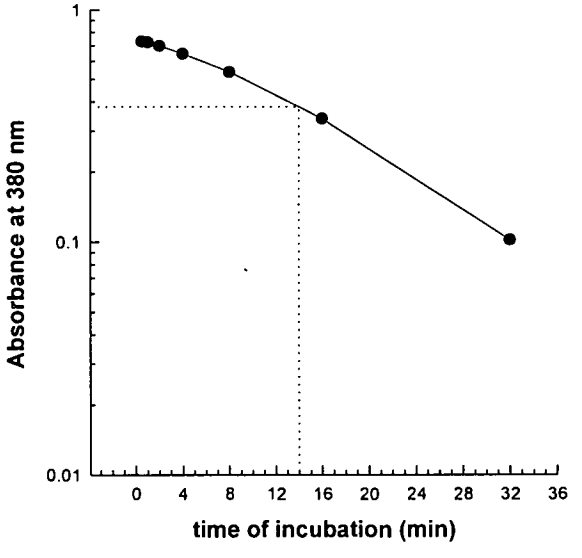


Fig. 3.16: Short-term kinetics of alkaline hydrolysis (§ 2.6.2) of compound B. A_{380} indicates remaining compound B.

3.5.3.2 Test for multiple esters

To test for multiple esters in compound B, partial saponification of ^{3}H-labelled compound was carried out (see also discussion). However, no intermediate product was formed (Fig. 3.17).

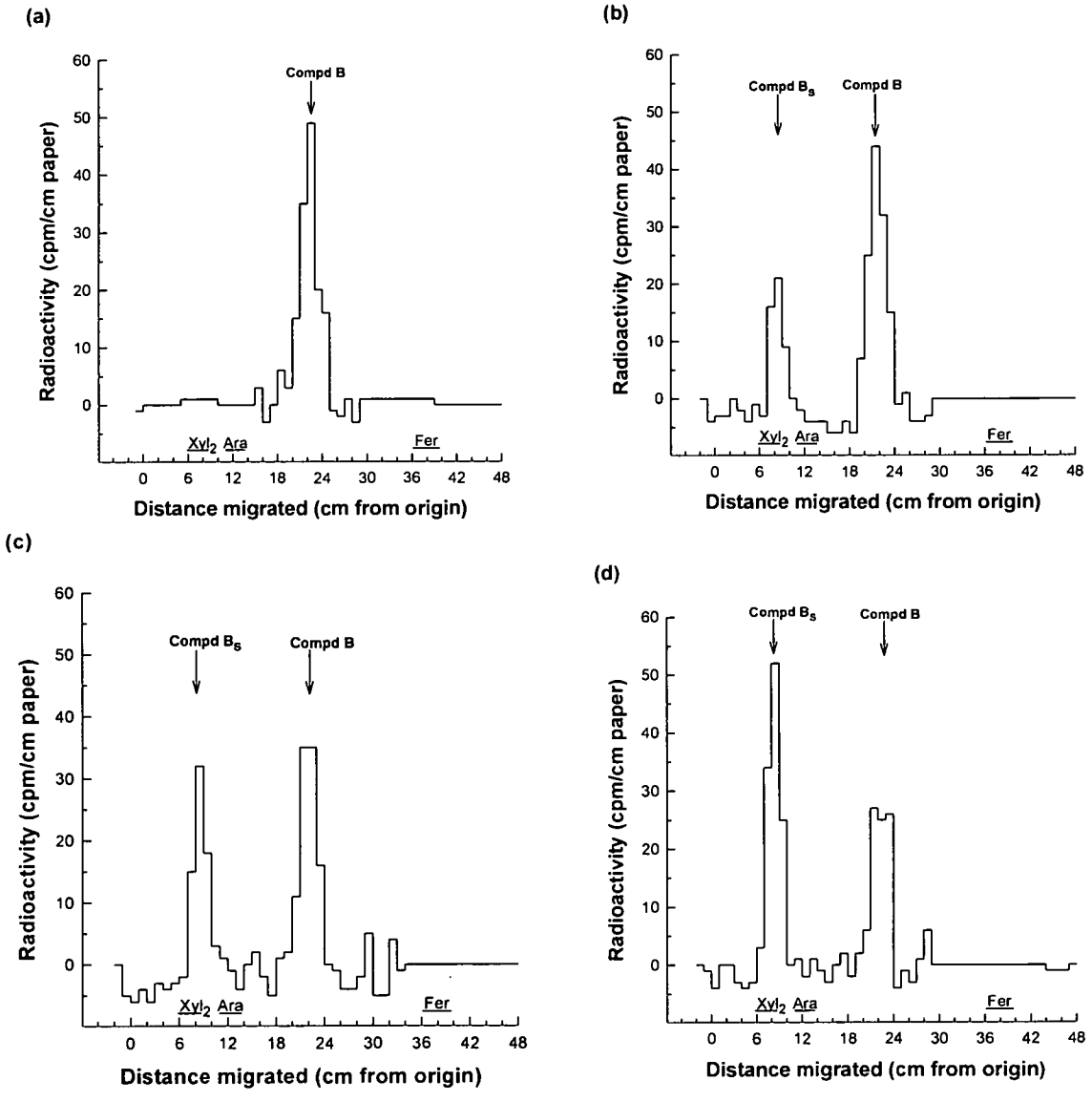


Fig. 3.17: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of ^{3}H-labelled compound B formed after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min. Xyl_2, Ara and Fer were used as external markers. "Compd B" and "compd B_S" show their approximate position (Table 3.1).

3.5.4 Monosaccharide composition

A portion of ^{3}H-labelled compound B_S was incubated with 2 M TFA (§ 2.6.1) and chromatographed by PC in EPW_1 (§ 2.5.1). Figure 3.18 shows two radioactive products.

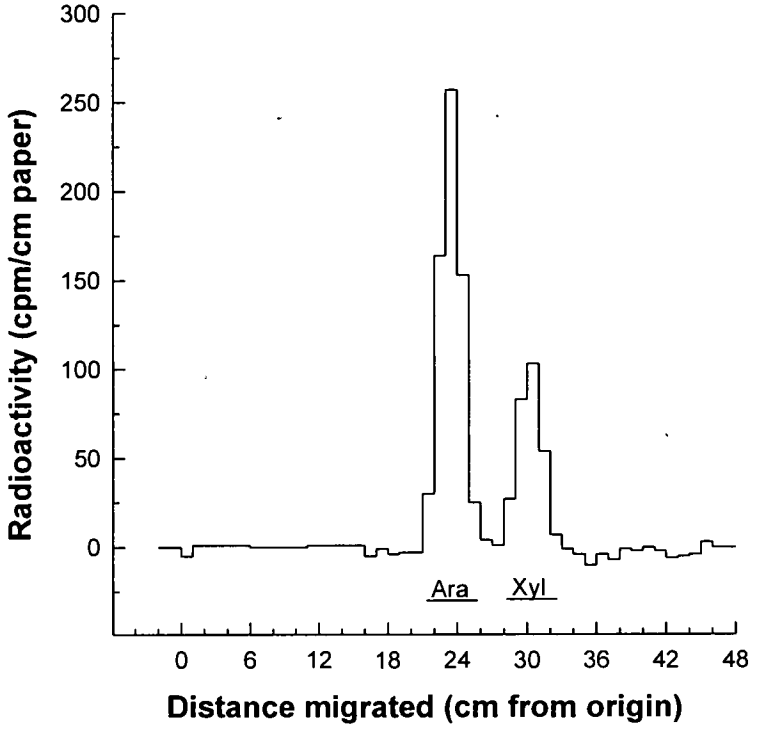


Fig. 3.18: PC in EPW_1 (§ 2.5.1) of severe acid hydrolysis (§ 2.5.1) products of ^{3}H-labelled compound B_S. Ara and Xyl were used as external and internal markers.

It is clear that compound B_S contains [^{3}H]arabinose and [^{3}H]xylose by reference to non-radioactive arabinose and xylose used as external and internal markers. The counts per minute under the peaks (from figure 3.18) were:

- $\sum$ peak 1 (arabinose, 21 to 26 cm): 633 cpm;
- $\sum$ peak 2 (xylose, 27 to 32 cm): 276 cpm.

The [^{3}H]Ara/[^{3}H]Xyl ratio (2.29 : 1.00) initially suggests that compound B_S is a trisaccharide.

3.5.5 DP compound B_S

3.5.5.1 Calculation of the specific radioactivity of [^{3}H]arabinose and [^{3}H]xylose residues in the total AIR

Severe acid hydrolysis (§ 2.6.1) was conducted on a portion of ^{3}H-labelled AIR. The hydrolysate was subjected to PC in EPW_1 (§ 2.5.1). The arabinose and xylose (§ 2.6.8) were eluted and assayed ① for ^{3}H by scintillation counting (Table 3.2) in TS (§ 2.7.1.2) and ② for reducing sugars (Table 3.4) by the PAHBAH-assay (§ 2.7.2) using L-arabinose and D-xylose for standard curves (Fig. 3.19).

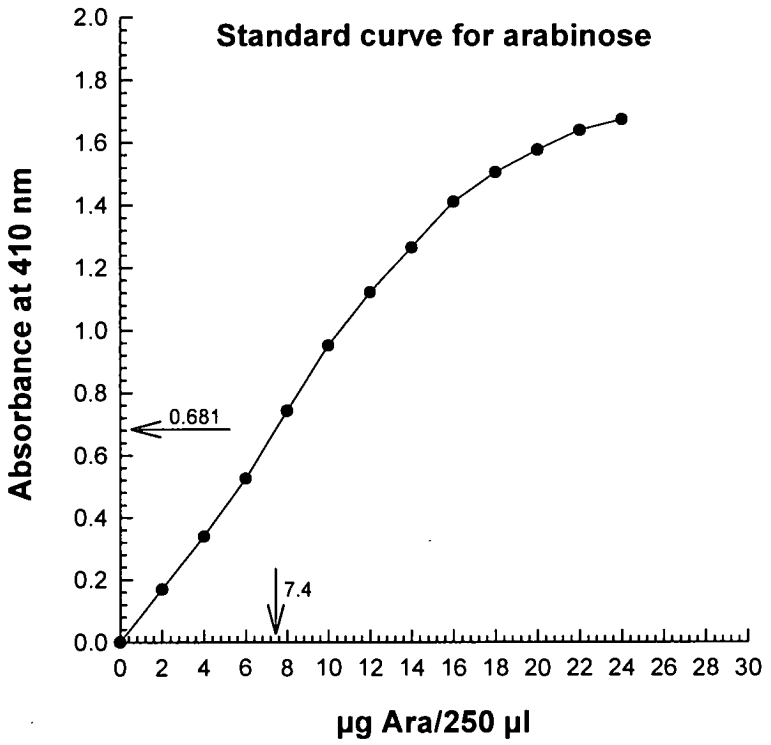
Aliquot No.	blank cpm/33 µl	H#	arabinose cpm/33 µl	H#	xylose cpm/33 µl	H#
1	22	86.4	2108	87.1	1178	86.9
2	21	87.0	2462	86.3	1146	86.9
3	21	86.0	2340	85.4	1160	86.9
4	20	85.1	2321	86.4	1234	86.7
5	21	85.1	2214	86.4	1145	86.1
6	27	88.2	2357	85.9	1174	86.9
$\bar{x}$	22	86.3	2300	86.3	1173	86.7
σ_{n-1}	2.6	1.3	129	0.6	35	0.3

Table 3.2: Blank, [^{3}H]Ara and [^{3}H]Xyl were assayed for radioactivity. Six 33-µl aliquots of out of 5 ml (§ 2.6.8) were taken, H# = quench number.

The total radioactivity was 50.0 MBq for L-arabinose and 25.4 MBq for D-xylose per 5 ml eluate (Table 3.3).

Parameter	arabinose	xylose
$\bar{x}$ sugar (cpm/33 µl)	2300	1173
$\bar{x}$ blank(cpm/33 µl)	22	22
corrected (cpm/33 µl)	2278	1151
% efficiency (from ^{3}H-quench-curve)	41.4	41.3
dpm/33µl	5500	2790
kBq/33µl	330.2	167.4
total MBq in 5 ml	50.03	25.36

Table 3.3: Calculation of total radioactivity per 5 ml eluate.



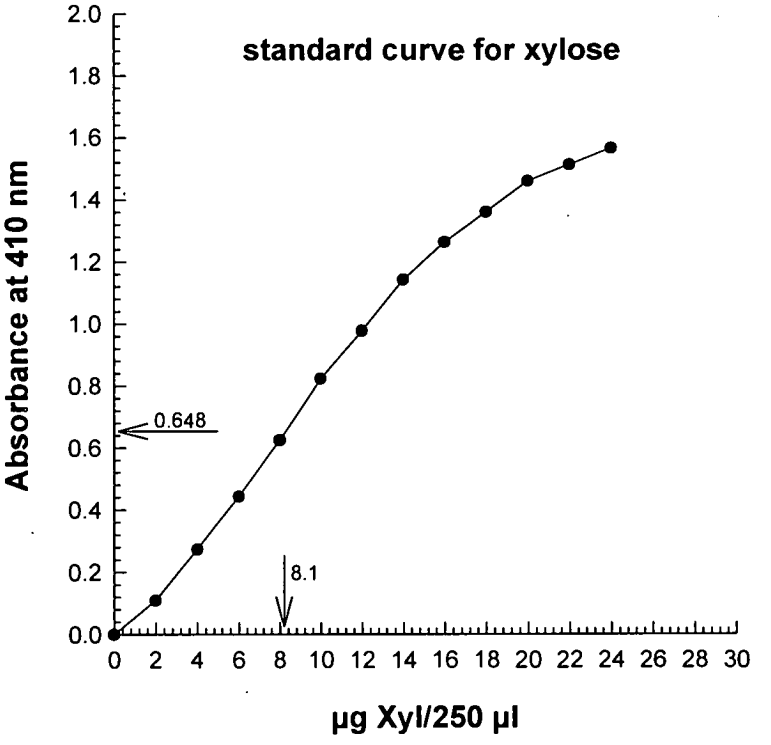


Fig. 3.19: Standard curves for PAHBAH assay of L-arabinose and D-xylose previously dried in a thermostat vacuum oven over phosphorus pentoxide for 9 hours. Each point is an average of 4 assays.

Repetition No	absorbance at 410 nm		
	blank	arabinose (1 : 10)	xylose (1 : 10)
1	0.091	0.697	0.732
2	0.082	0.753	0.710
3	0.079	0.717	0.710
4	0.089	0.757	0.727
5	0.081	0.762	0.702
6	0.061	0.775	0.710
7	0.064	0.764	0.723
8	0.072	0.776	0.739
9	0.067	0.755	0.755
10	0.074	0.812	0.738
$\bar{x}$	0.076	0.757	0.725
σ_{n-1}	0.007	0.023	0.012

Table 3.4: PAHBAH-assay. Ten 33-µl aliquots of blank, [^{3}H]Ara and [^{3}H]Xyl out of 5 ml eluate were assayed for reducing sugar.

The specific radioactivity of [^{3}H]Ara and [^{3}H]Xyl in the total AIR were 11.17 MBq/mg and 5.17 MBq/mg, respectively (Table 3.5). Thus, the ratio arabinose/xylose in compd B_S can be estimated as 1.06 : 1.00 (0.205/0.1934) - assuming that the specific activities of the pentose moieties in this compound are the same as in the whole cell wall. Therefore it can be concluded that compound B_S is a disaccharide of arabinose and xylose.

Parameter	arabinose	xylose
A_{410} from PAHBAH-assay	0.757	0.725
A_{410} of blank	0.076	0.076
Δ (A_{410})	0.681	0.648
Pentose from standard curves in µg/33µl (Fig. 3.19)	7.40	8.10
total mg in 5 ml eluate	4.48	4.91
(a) spec. activity in total AIR (MBq/mg)	11.17	5.17
(b) ratio (Ara : Xyl) in B_S on ^{3}H-basis (§ 3.5.4)	2.29	1.0
(b)/(a)	0.2050	0.1934

Table 3.5: Calculation of the ratio of [^{3}H]arabinose and [^{3}H]xylose by use of the specific radioactivity.

3.5.5.2 Gel-permeation chromatography on Bio-Gel P-2 of compound B_S

The DP of compound B_S was tested by gel-permeation chromatography on Bio-Gel P-2 with internal marker mono-, di- and trisaccharides (§ 2.5.5). By reference to the internal marker sugars, fractions 45 to 49 contained disaccharides (Fig. 3.20). The ^{3}H-labelled compound B_S co-eluted exactly with maltose (fractions 45, 46 and 47) although some fractions outside of the ^{3}H-peak contained marker xylobiose. The most plausible conclusion is that compound B_S is a disaccharide.

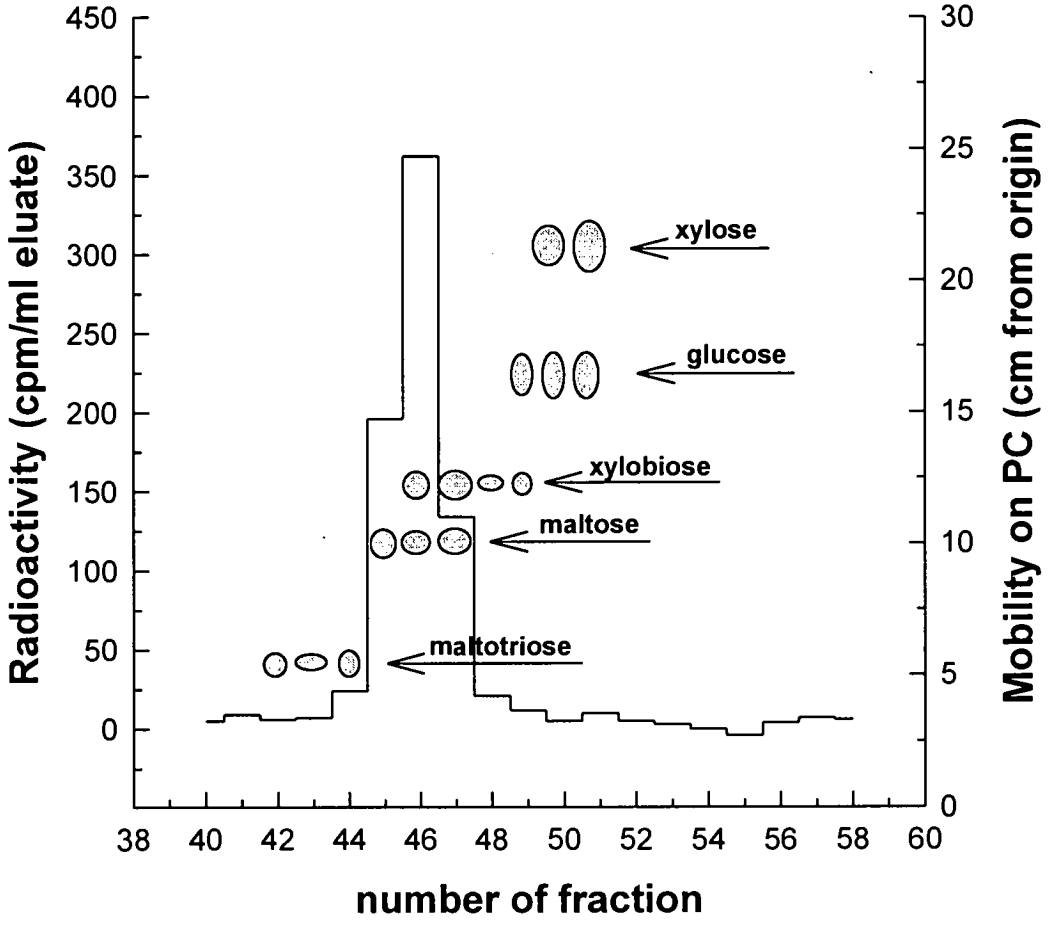


Fig. 3.20: Behaviour of ^{3}H-labelled compound B_S on Bio-Gel P-2. Fractions 40 to 58 were subjected to PC in BAW (§ 2.5.1) using maltotriose, maltose, xylobiose, glucose and xylose as external markers (mobility on PC) and assayed for radioactivity (§ 2.7.1.2).

3.5.6 Examination of the reducing terminus of compound B_S

3.5.6.1 Paper chromatography

A portion of compound B_S was reduced with sodium borohydride (§ 2.6.6) in an attempt to yield compound B_S-ol followed by complete acid hydrolysis (§ 2.5.1). Paper chromatography in BAB (§ 2.5.1) showed that the reduction and the hydrolysis had been successful (Fig. 3.21). Two main radioactive products were produced: xylose and xylitol/arabinitol. Since we know (§ 3.5.5) that compound B_S is a disaccharide of arabinose and xylose, the second peak (26 to 30 cm from the origin) should be arabinitol. It can thus be suggested that arabinose is the reducing terminus of compound B_S.

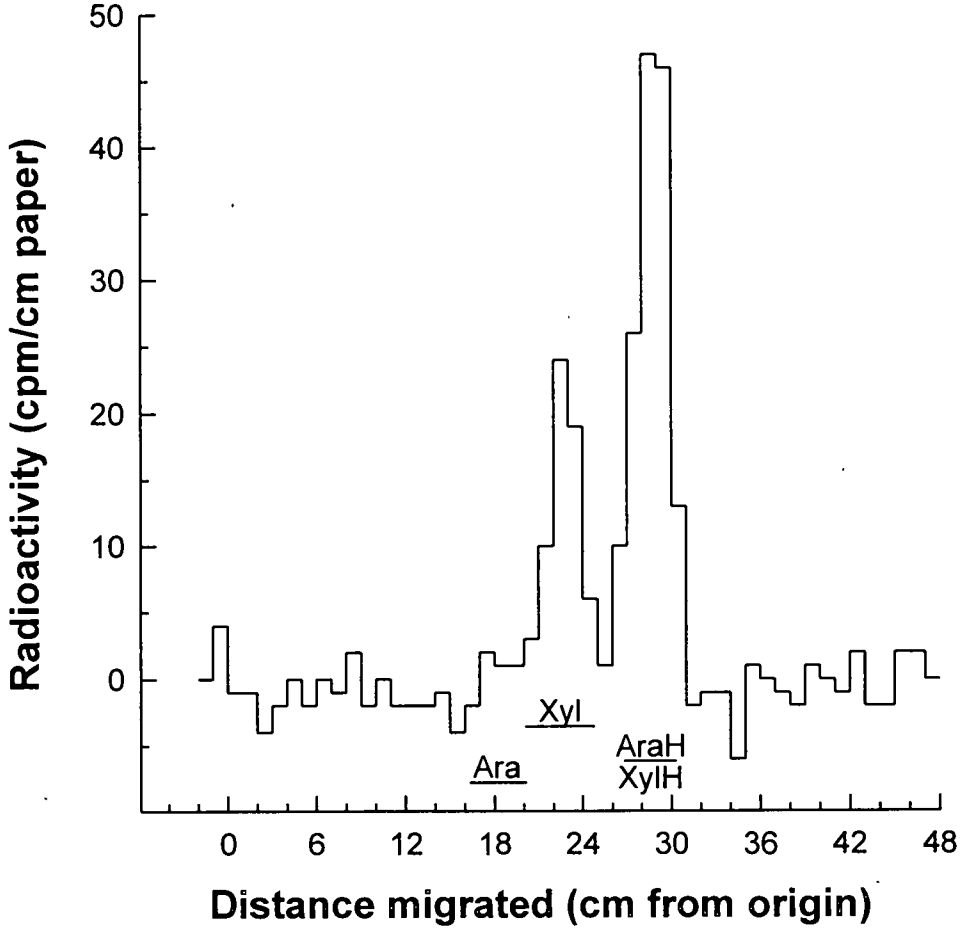


Fig. 3.21: PC in BAB (§ 2.5.1) of complete acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compound B_S-ol. Xyl, XylH, Ara and AraH were used as external markers.

3.5.6.2 Paper electrophoresis

Separation of AraH and XylH was achieved by paper electrophoresis in borate buffer at pH 9.4 (Fig. 3.22). The internal markers arabinitol and xylose corresponded exactly with the radioactive products which had been formed.

From these data it can be concluded that compound B_S is a disaccharide made of arabinose and xylose, with arabinose as the reducing component.

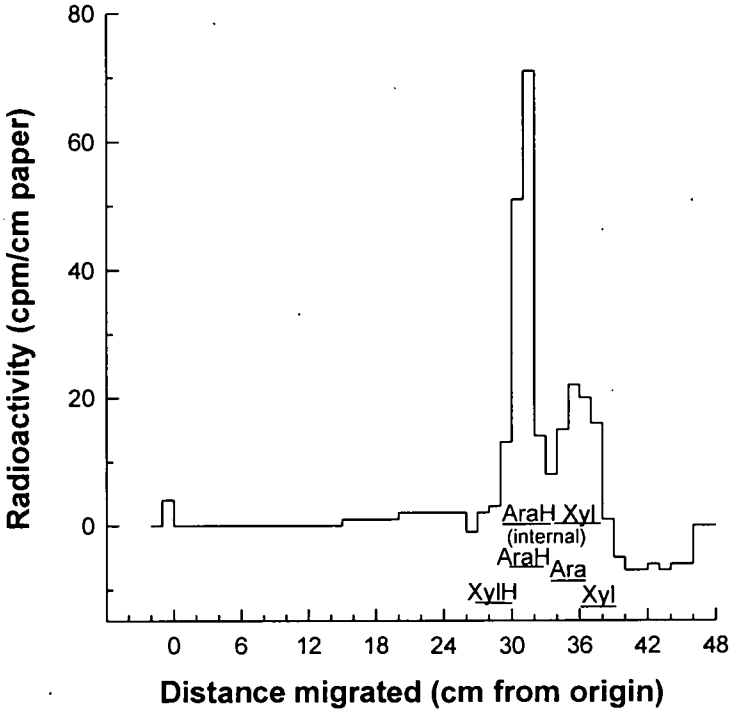


Fig. 3.22: PE in borate buffer (§ 2.5.2) of hydrolysis products of ^{3}H-labelled compound B_S-ol. AraH and Xyl were used as external and internal and Ara and XylH as external markers.

3.5.7 Linkage within the disaccharide

Assuming that the arabinose moiety of compound B_S had been 5-*O*-feruloylated and furanosidically linked to the xylan backbone, the Xyl→Ara linkage of compd B_S would have to be (1→2) or (1→3). These possibilities were examined by paper electrophoresis and by Smith degradation.

3.5.7.1 Paper electrophoresis

A portion of ^{3}H-labelled compound B_S-ol was subjected to paper electrophoresis in molybdate buffer (Fig. 3.23) together with appropriate markers with (1→2) and (1→3)-linkages.

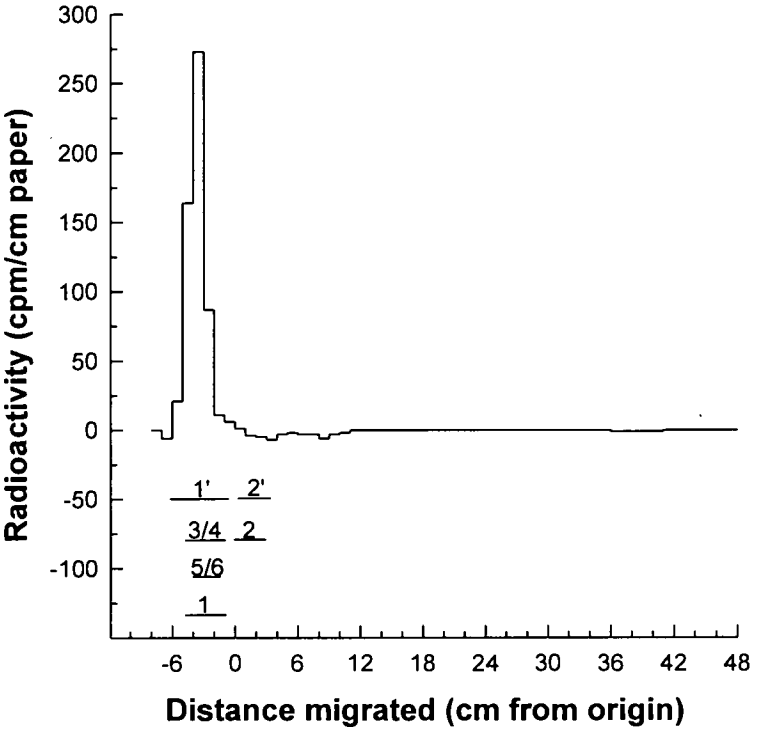


Fig. 3.23: PE in molybdate buffer (§ 2.5.2) of ^{3}H-labelled compd B_S-ol. 2-Deoxyarabinitol (1), D-galactosyl-β-1,3-D-arabinitol (2), methyl α-(3) and β-D-xyloside (4) and methyl α-(5) and β-D-galactoside (6) served as external markers and 1' and 2' (from 1 and 2, respectively) as internal markers.

Compound B_S-ol showed the same mobility as 2-deoxyarabinitol, so it can be assumed that both have a similar (lack of) ability to complex with molybdate. 3-*O*-Substituted arabinitol has a higher ability to bind molybdate, as shown by the greater mobility of Gal-(1→3)-AraH. Since 2-deoxyarabinitol is "blocked" at position *O*-2 it can be assumed that in B_S-ol the hydroxyl group at C-2 of the arabinitol moiety is substituted. Therefore a (1→2)-linkage between xylose and arabinose in compound B_S can be concluded.

3.5.7.2 Smith degradation

Another way of addressing the linkage between xylose and arabinose was $NaIO_4$-oxidation (§ 2.6.7) of ^3H-labelled compound B_S-ol. The major, non-volatile, radioactive product of the Smith degradation co-migrated with the external marker glycerol (Fig. 3.24).

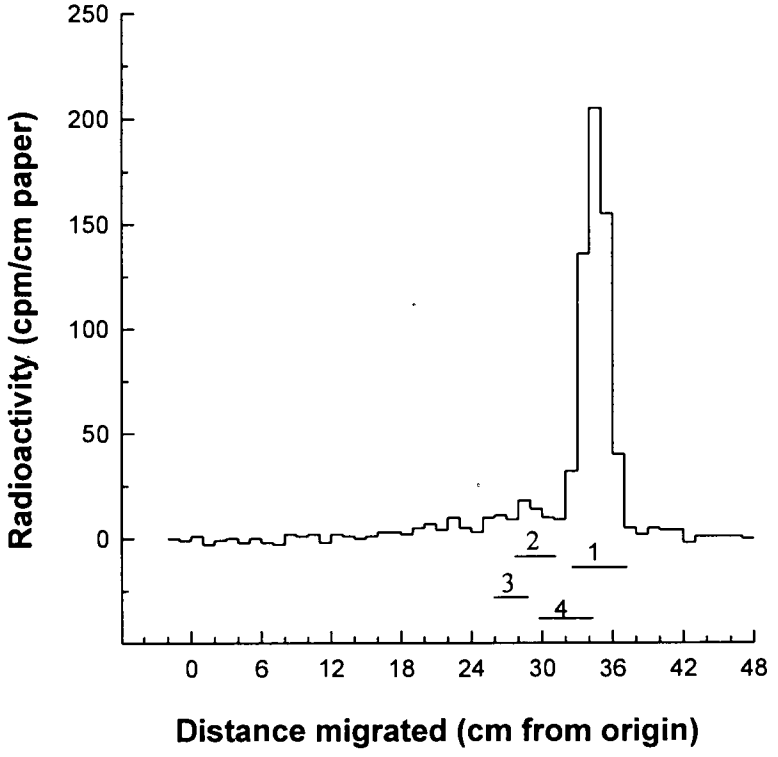


Fig. 3.24: PC in EAW (§ 2.5.1) of products of ^3H-labelled compd B_S-ol after $NaIO_4$-oxidation, $NaBH_4$-reduction and TFA hydrolysis. Glycerol (1), xylose (2), arabinose (3) and erythritol and threitol (4) were used as external markers.

The eluted ^3H-labelled product also co-migrated exactly with the internal marker glycerol on PC in EPW_1 (Fig. 3.25). These data strongly indicate that there is a (1→2)-linkage of xylose to arabinose within compound B_S. The ^3H-glycerol would arise from C_1, C_2 and C_3 of the [1-^3H]arabinitol. If the linkage had been (1→3), then non-radioactive glycerol would arise from C_2, C_3 and C_4 with the loss of the radiolabelled position (C_1) as [^3H]formaldehyde.

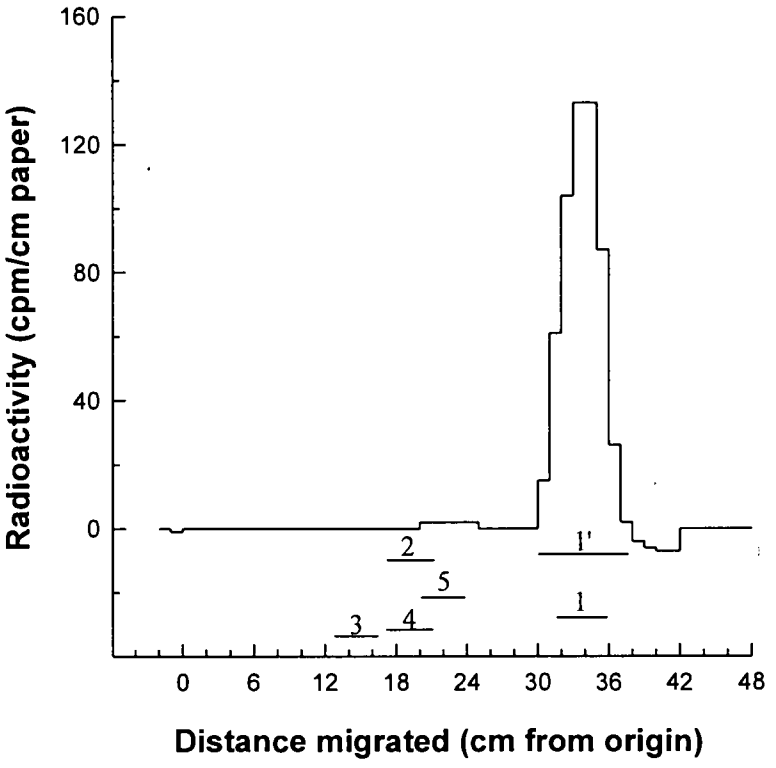


Fig. 3.25: PC in EPW_1 (§ 2.5.1) of ^3H-labelled product from figure 3.24. EryH (5), ThrH (4), arabinose (3), xylose (2) (all external) and glycerol (internal (1) and external (1')) as markers.

3.5.8 Configuration of the (1→2)-linkage

3.5.8.1 Digestion with β-xylosidase (from Sigma)

To test whether the (1→2)-linkage in compd B_S has a β-configuration, enzymic digestion of compound B with β-xylosidase was used (Fig. 3.26). The ultimate products were Ara/Xyl (not resolved in BAW), showing that the enzyme preparation was contaminated with feruloylesterase activity. All the intermediate products predicted of compound B (see discussion) were also observed. It seems that most of the conversions took place within the first 50 min.

Based on this result, it appears that the (1→2)-linkage within the molecule is in the β-configuration. However, the possibility cannot be ignored that the break-down of compound B is due to α-xylosidase activity (possibly present in the enzyme solution). To examine this, a substrate in α-configuration was tested.

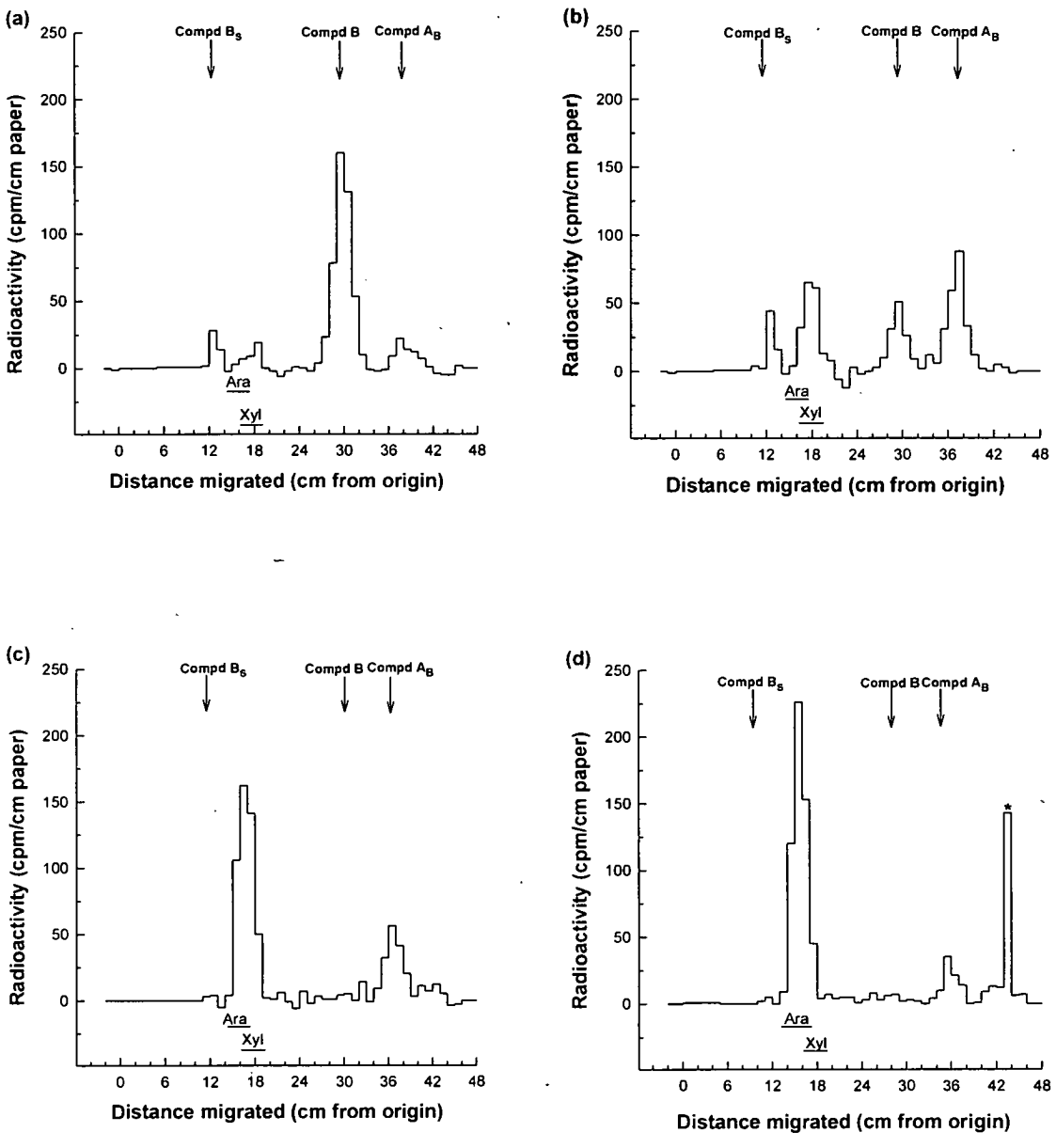


Fig. 3.26: PC in BAW (§ 2.5.1) of digestion products of ^{3}H-labelled compound B with β-xylosidase (from Sigma) formed after 10 min (a), 50 min (b), 250 min (c) and 1250 min (d). Ara and Xyl were used as external markers. The strip marked (*) is presumed to be contaminated with extraneous ^{3}H (genuine radioactive sugars have never been observed to migrate as such a sharp "peak"). "Compd B" and "compd B_S" show their approximate position (Table 3.1).

3.5.8.2 Enzymic digestion with a test substrate in α-configuration

To test whether the enzyme solution contained any α-xylosidase, a portion of ^{3}H-labelled isoprimeverose (xylosyl-glucose with an α(1→6)-linkage; see also § 2.3.2.2) was treated with Sigma β-xylosidase solution (§ 2.6.3.2) (Fig. 3.27).

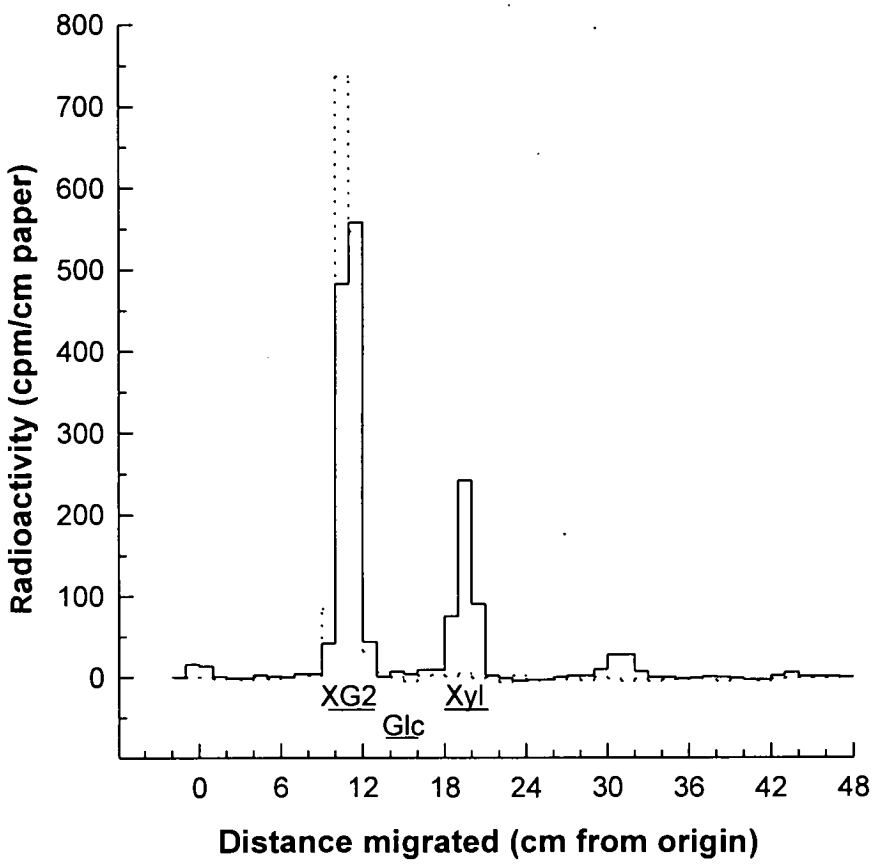


Fig. 3.27: PC in BAW (§ 2.5.1) of digested ^{3}H-labelled isoprimeverose by "β-xylosidase". ..... = untreated XG2, — = XG2 + enzyme solution. Xyl, Glc and XG2 were used as external markers.

Unfortunately, the enzyme solution clearly contained α-xylosidase activity (as well as esterase). Within 50 min approximately 26% of the α-xylosyl bonds had been hydrolysed.

3.5.9 Position of the ester-linkage

To test whether the feruloyl group of compound B is linked to *O*-5 of arabinose, ^{14}C-labelled compound A_B (derived from compound B by digestion in "β-xylosidase" from Sigma (see figure 3.26)) was subjected to PC with internal authentic 5-*O*-feruloyl-L-arabinose (from maize). Figure 3.28 shows that the ^{14}C-compound A_B corresponded with the internal standard of 5-*O*-feruloyl-L-arabinose of maize in all solvents investigated.

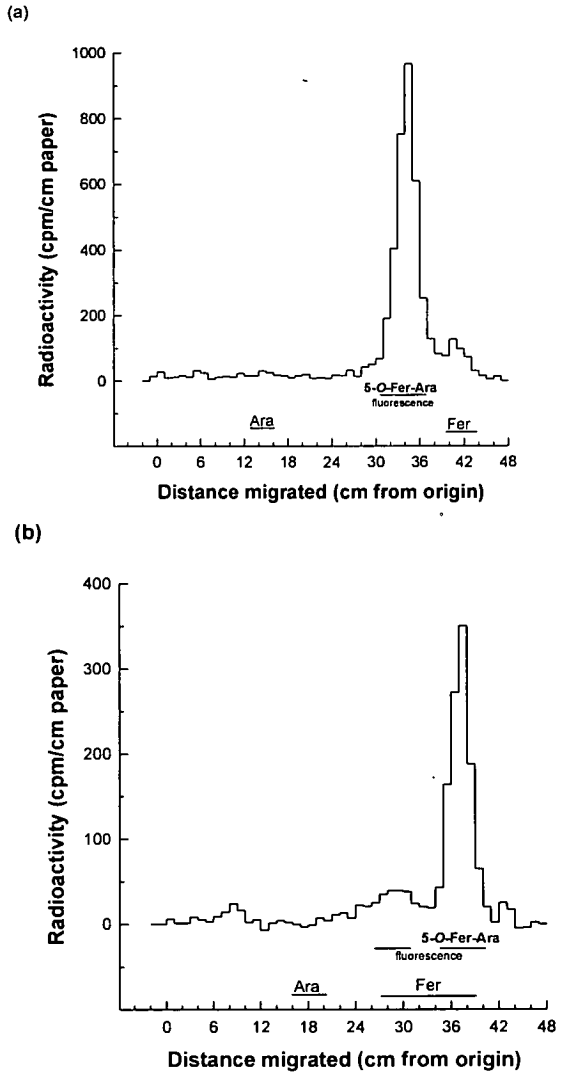


Fig. 3.28: Co-migration of ^{14}C-labelled compound A_B (derived from ^{14}C-compound B by "β-xylosidase" from Sigma) with internal marker authentic 5-*O*-feruloyl-L-arabinose (of maize). PC's were developed in (a) BAW, (b) BEW, (c) EPW_1 and (d) ΦW, and the PE (e) in borate buffer (§ 2.5.1). Ara and Fer were used as external markers.

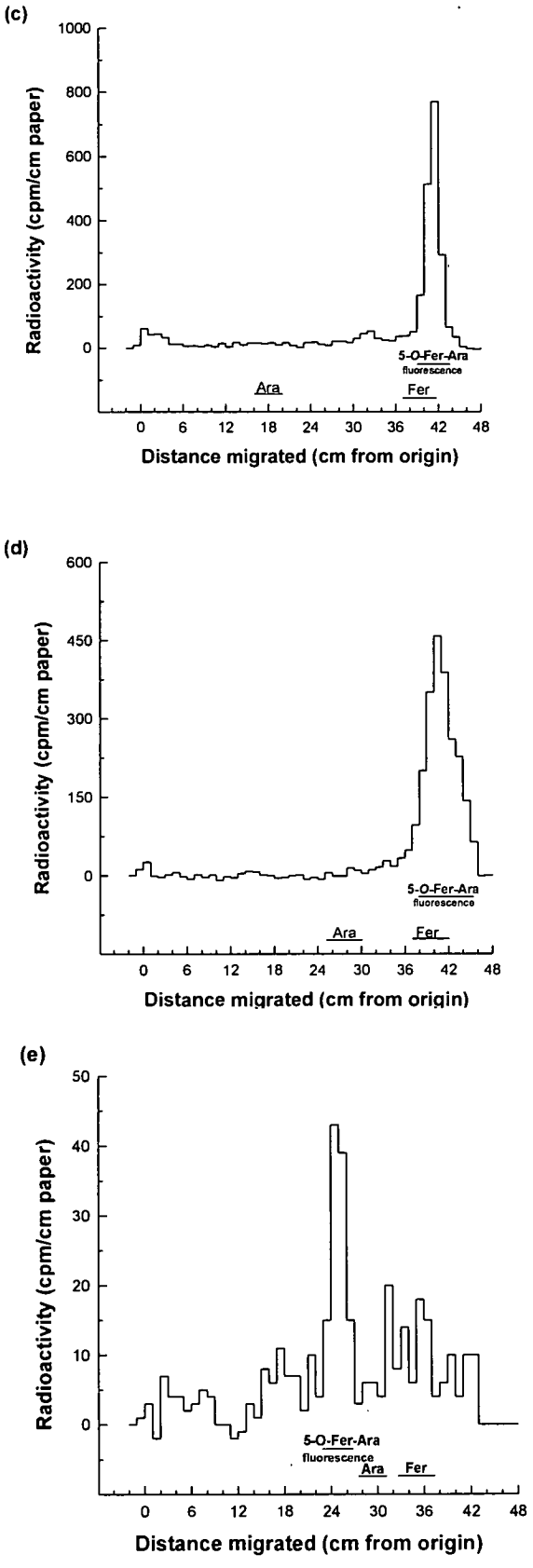


Fig. 3.28 continued.

To test whether the absorbance profile at 323 nm (characteristic of a feruloyl ester at neutral pH; § 3.3) corresponds exactly with the ^{14}C profile, the material in strips 24 to 45 cm (from the origin, figure 3.28 (b) after removal of scintillant) was eluted and the absorbance measured (Fig. 3.29).

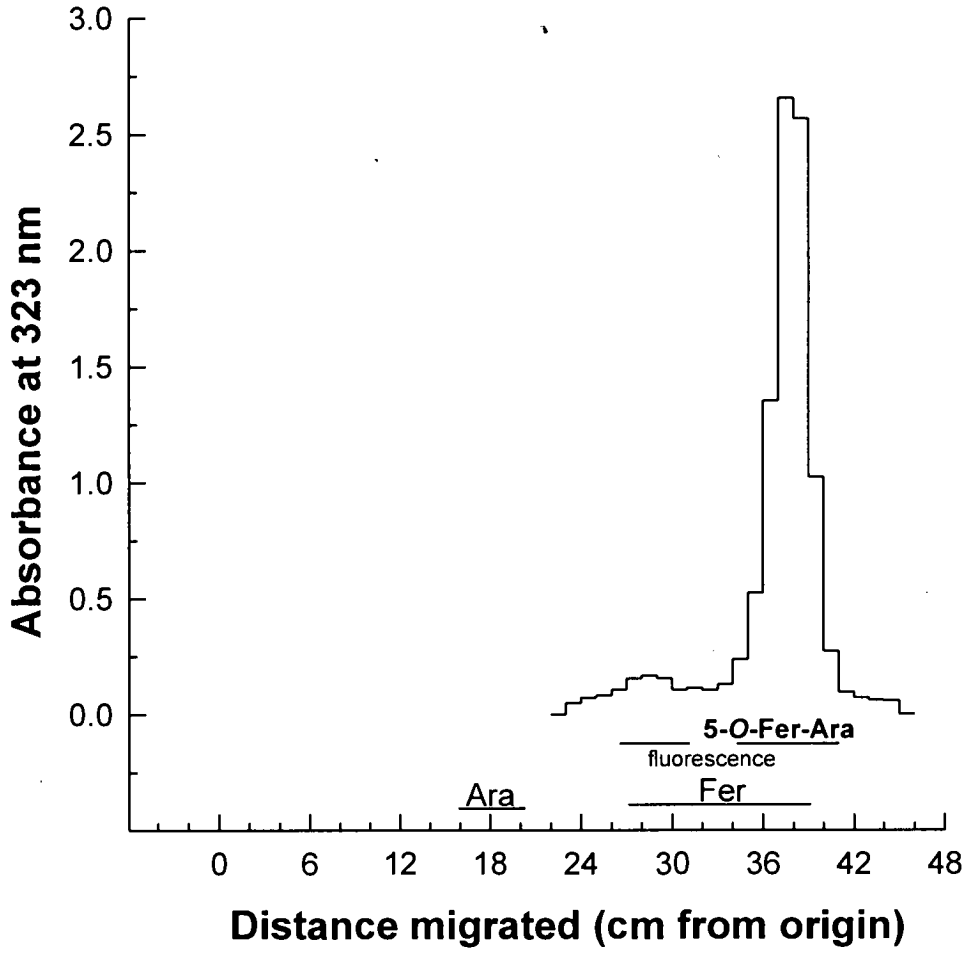


Fig. 3.29: A_{323} of eluates from strips of the BEW-PC shown in figure 3.28 (b) (24 to 45 cm from origin) after scintillation-counting.

The absorbance profile of figure 3.29 corresponds with the ^{14}C profile of compound A of figure 3.28 (b). It can thus be concluded that compound A_B of fescue is 5-*O*-feruloyl-L-arabinose.

3.5.10 NMR- and IR-spectroscopy

To corroborate the structure of compound B, analysis by ^{1}H- and ^{13}C-NMR spectroscopy and methylation analysis (§ 3.5.11) were carried out.

3.5.10.1 Purification of compound B by HPLC

A portion of compound B that had been partially purified by reverse-phase column chromatography (Mega BondElut) and PC in BAW followed by PC in BEW, was applied on to an analytical reversed-phase HPLC column (§ 2.5.3) together with a portion of ^{14}C-labelled compound B and eluted with a H_2O/MeCN-gradient of 20→50% for 40 min. The eluate was monitored at 280 nm (Fig. 3.30 (a)), fractions were collected and assayed for radioactivity (§ 2.7.1.2) (Fig. 3.30 (b)). The profile of ^{14}C-labelled compound B reveals that the major peak in figure 3.30 (a) (retention time of 5.78 min) contains compound B. Subsequently the gradient was optimised to a H_2O/MeCN-gradient of 15→25%.

Based on this result, 3.5 mg pure compound B was prepared by using a preparative reverse-phase HPLC column under the same condition as described above. Fractions containing compound B (retention time of 15.07 in figure 3.31) were collected and dried *in vacuo* and taken for NMR and methylation analysis.

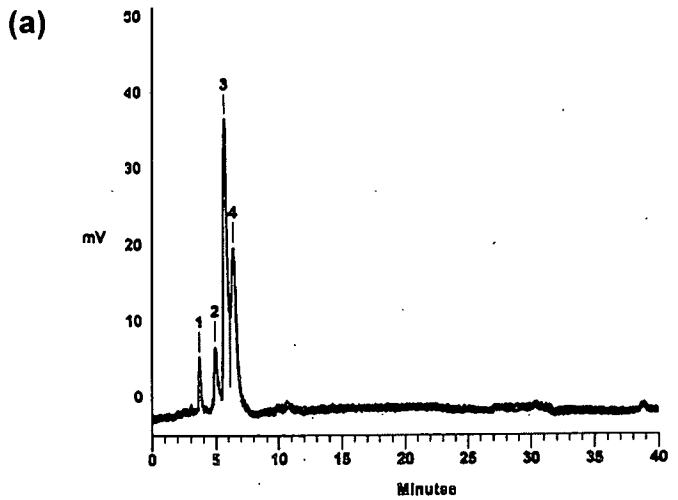


Fig. 3.30: HPLC of partially purified compound B on an analytical reverse-phase HPLC column in a H_2O/MeCN-gradient of 20→50% over 40 min. The eluate was monitored at 280 nm (a); fractions were collected and assayed for radioactivity (b) (§ 2.7.1.2).

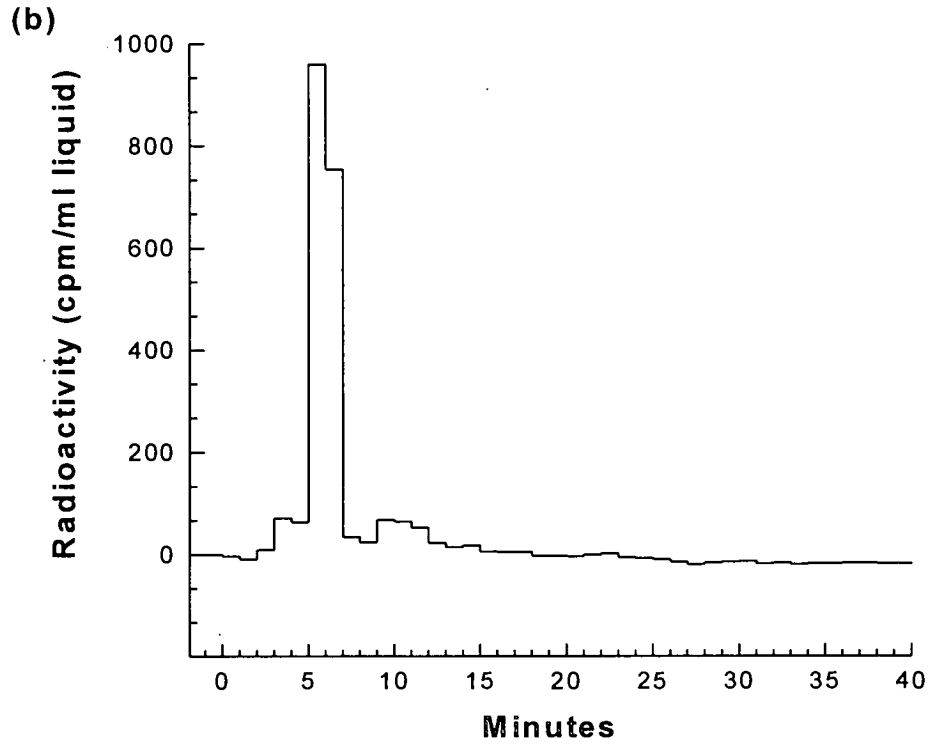


Fig. 3.30 continued.

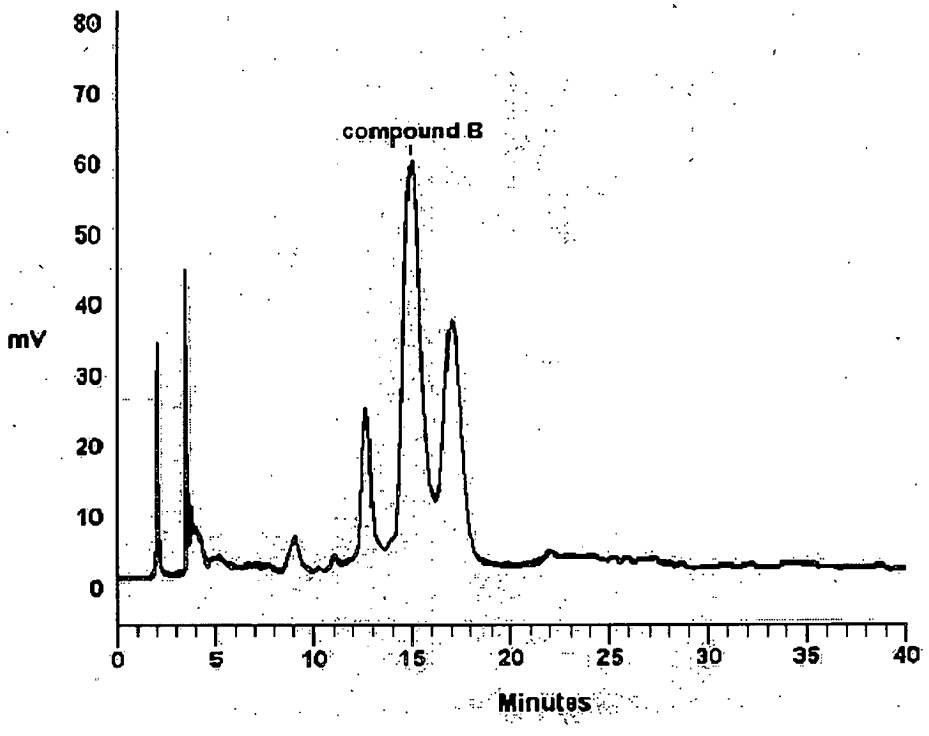


Fig. 3.31: HPLC of partially purified compound B on a preparative reverse-phase column in a H_2O/MeCN-gradient of 15→25% over 40 min. The eluate was monitored at 280 nm.

3.5.10.2 NMR analysis

NMR spectra of purified compound B (3.5 mg) were acquired in D_2O at 300.13 MHz for ^1H (Fig. 3.32 (a)) and at 75.46 MHz for ^{13}C (Fig. 3.32 (b)).

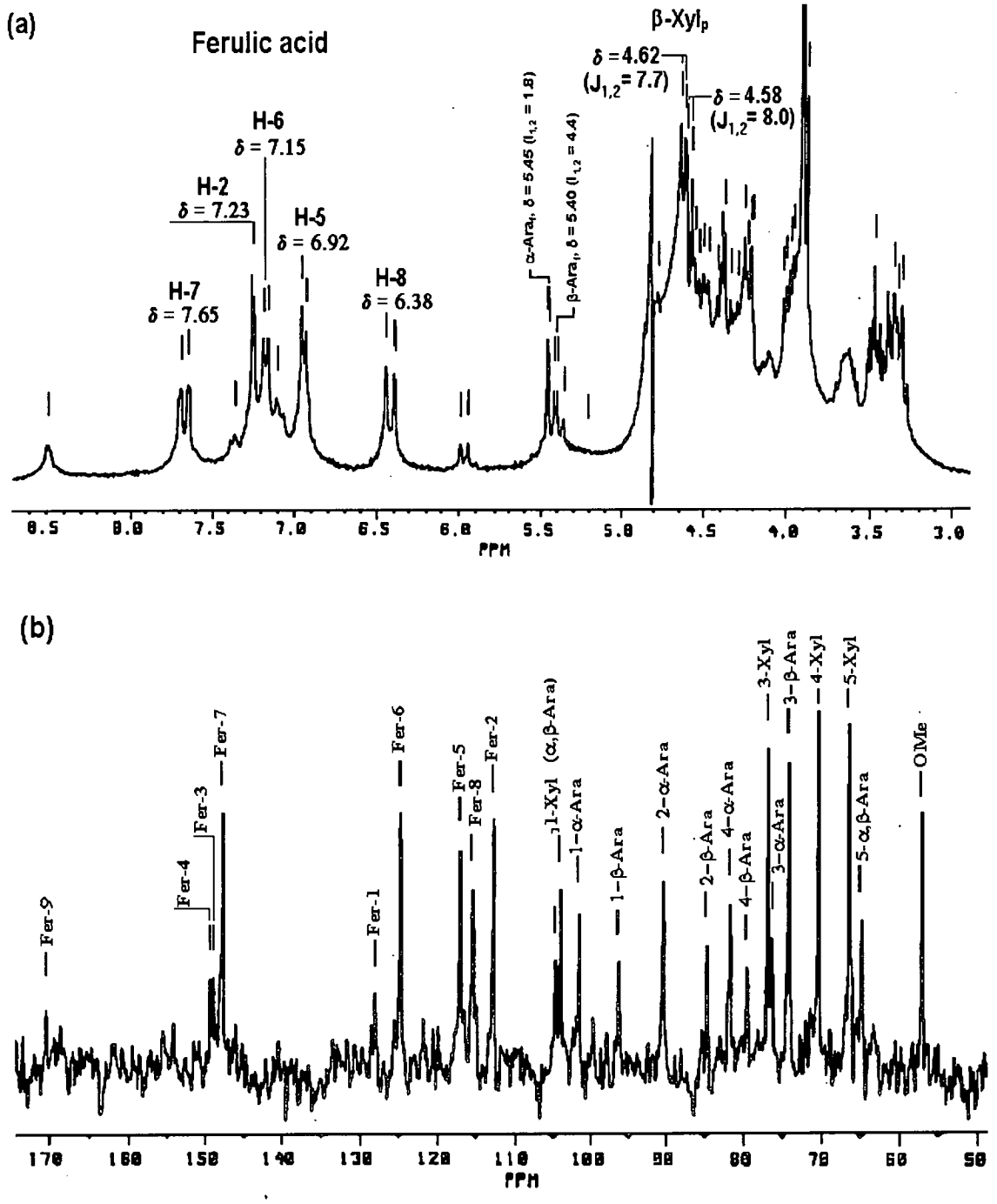


Fig. 3.32: NMR spectra of purified compound B were acquired in D_2O at 300.13 MHz for ^1H (a) and at 75.46 MHz for ^{13}C (b).

In the ^1H spectrum (Fig. 3.32 (a)), anomeric signals of α-Ara$_f$ and β-Ara$_f$ were found at δ 5.45 ($J_{1,2}$ 1.8) and 5.40 ($J_{1,2}$ 4.4), respectively, because of α, β equilibration of the Ara$_f$ residue at the reducing end of the feruloylated disaccharide. Two H-1 signals of β-Xyl$_p$ were also detected; these were identified on the basis of $J_{1,2}$ coupling constant values at δ 4.62 ($J_{1,2}$ 7.7) and 4.58 ($J_{1,2}$ 8.0). The assignment of the ferulate ester signals (analysed in collaboration with Dr. Farkaš) is given in Table 3.6 and agree with those published by Angyal (1979), Himmelsbach *et al.* (1994) and Seriani & Baker (1984).

	^1H chemical shift (ppm) and coupling constants (Hz)					
Residue	δ H-1 (J)	δ H-2 (J)	δ H-5 (J)	δ H-6 (J)	δ H-7 (J)	δ H-8 (J)
α-Ara$_f$	5.45 (1.8)					
β-Ara$_f$	5.40 (4.4)					
β-Xyl$_p$	4.62 (7.7)					
β-Xyl$_p$	4.58 (8.0)					
Fer		7.23	6.92	7.15	7.65	6.38

Table 3.6: Assignment of ^1H NMR spectrum of the ferulic acid ester signals and anomeric signals of α-Ara$_f$, β-Ara$_f$ and β-Xyl$_p$.

In the anomeric region of the ^{13}C-NMR spectrum, four signals were observed: two signals of β-D-Xyl$_p$ residue were present at δ 104.46 and 103.82, again due to an α, β-equilibrium of the arabinose unit. C-1 resonances of α- and β-L-Ara$_f$ unit appeared at 101.37 and 96.18, respectively. The suggested β-Xyl$_p$-(1→2)-Ara$_f$ linkage (Fig. 3.13, §§ 3.5.7 and 3.5.8) was confirmed by the characteristic glycosylation downfield shift of the C-2 α- and β-Ara$_f$ signals to δ 90.24 and 84.57, respectively. The assignment of all carbon signals including ferulic ester (analysed in collaboration with Dr. Farkaš) is given in Table 3.7 and agree with those published (see above).

	^{13}C Chemical shifts (δ, ppm)									
Residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	O-Me
α-Ara$_f$	101.37	90.24	76.18	81.58	64.77	nd	nd	nd	nd	nd
β-Ara$_f$	96.18	84.57	74.07	79.45	64.77	nd	nd	nd	nd	nd
β-Xyl$_p$	104.16	74.07	76.73	70.32	66.35	nd	nd	nd	nd	nd
β-Xyl$_p$	103.82									
Fer	127.80	112.47	148.80	149.23	116.78	124.57	147.59	115.13	170.20	57.04

Table 3.7: Assignments of ^{13}C NMR spectrum of the ferulic acid ester signals and the signals of α-Ara$_f$, β-Ara$_f$ and β-Xyl$_p$. Methanol was used as internal reference (50.15 ppm with respect to tetramethylsilane).

3.5.10.3 FT-IR analysis

An IR-spectrum of HPLC-purified compound B (Fig. 3.33) on an NaCl plate was recorded between 4000 and 600 cm^{-1}. Table 3.8 shows the tentative assignments.

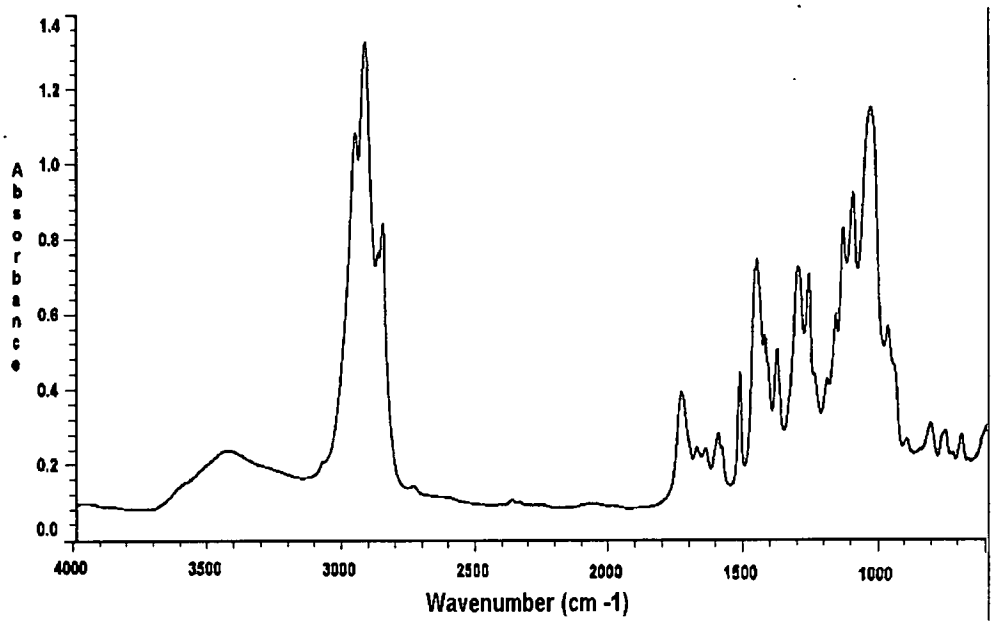


Fig. 3.33: FT-IR spectrum of HPLC-purified compound B on an NaCl plate.

ν (cm^{-1})	Tentative assignment	ν (cm^{-1})	Tentative assignment	ν (cm^{-1})	Tentative assignment
3427	OH	1578	ring, Ar	1136	nd
2959	CH	1514	ring, Ar	1101	nd
2925	CH_2	1461	CH	1040	C-O sacch.
2871	CH	1456	CH	969	nd
2853	CH_3	1423	nd	892	C(1)H sacch.
1731	C=O	1377	nd	806	C-H, out of plane, Ar
1672	C=C	1303	nd	751	C-H, out of plane, Ar
1651	C=C	1263	nd	721	C-H, out of plane, Ar
1638	HOH	1192	nd	690	nd
1592	C=C, Ar	1161	nd	666	nd

Table 3.8: Tentative assignments from the FT-IR spectrum of HPLC-purified compound B.

3.5.11 Methylation analysis

Methylation analysis is one of the fundamental methods used to study glycosidic linkages (Avigat, 1990), e.g. in disaccharides, in this case purified compound B. The most commonly used method is that of Hakomori (1964): treatment of DMSO with sodium hydroxide generates a methylsulphinyl carbanion as the alcoxide-forming permethylation reagent. The permethylated glycoside is hydrolysed, reduced, per-*O*-acetylated (§ 2.6.11) and the partially methylated alditol acetates are analysed by GC (Fig. 3.34).

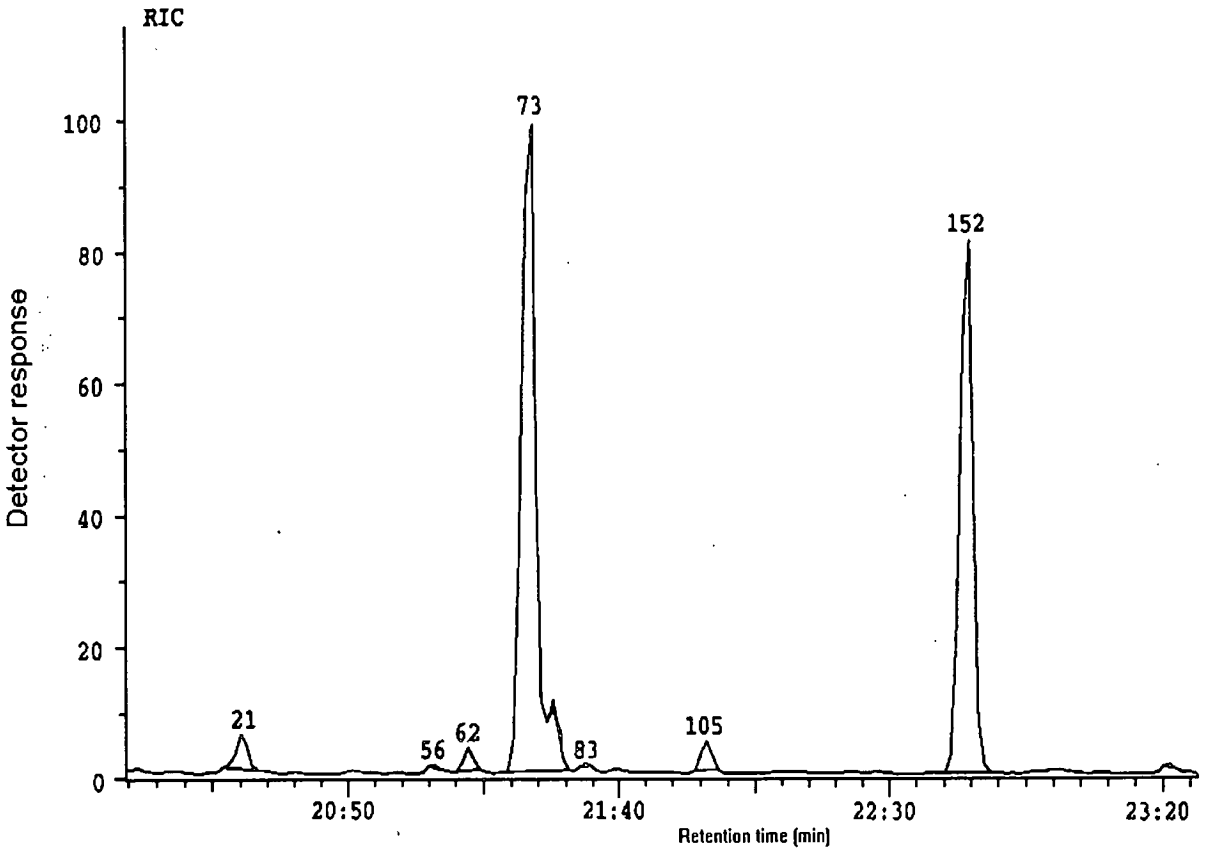


Fig. 3.34: GC of products of compound B after permethylation, acid hydrolysis, reduction and peracetylation. RIC = relative ion current.

Gas chromatography of the products of compound B after methylation analysis gave two major peaks (peak 73 and peak 152) (Fig. 3.34), which were further analysed by mass spectrometry (Fig. 3.35). Figure 3.36 shows possible structures.

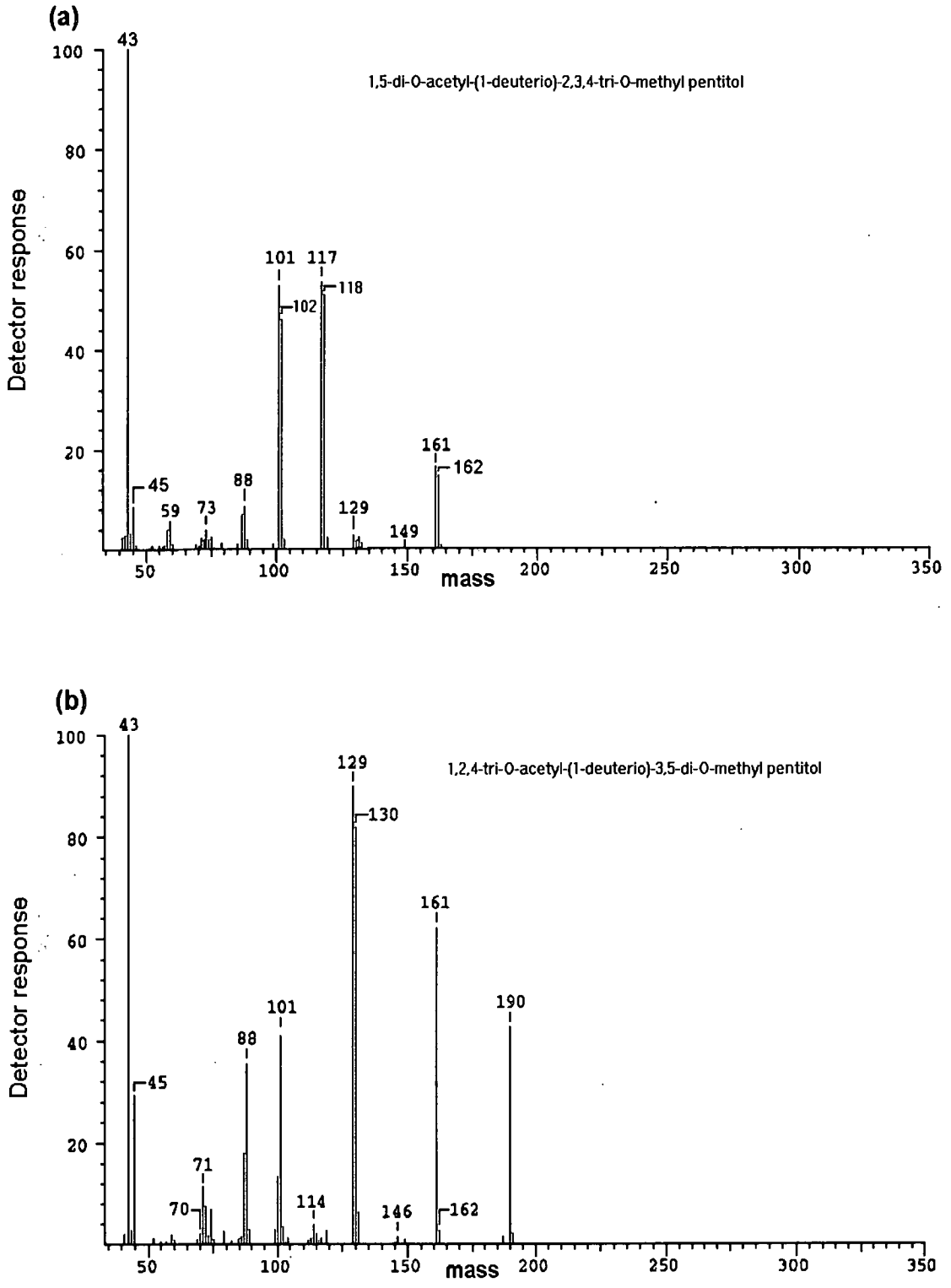


Fig. 3.35: Mass spectrometry of methylated alditol acetates obtained from compound B after GC from peak 73 (a) and peak (b) 152 of figure 3.34.

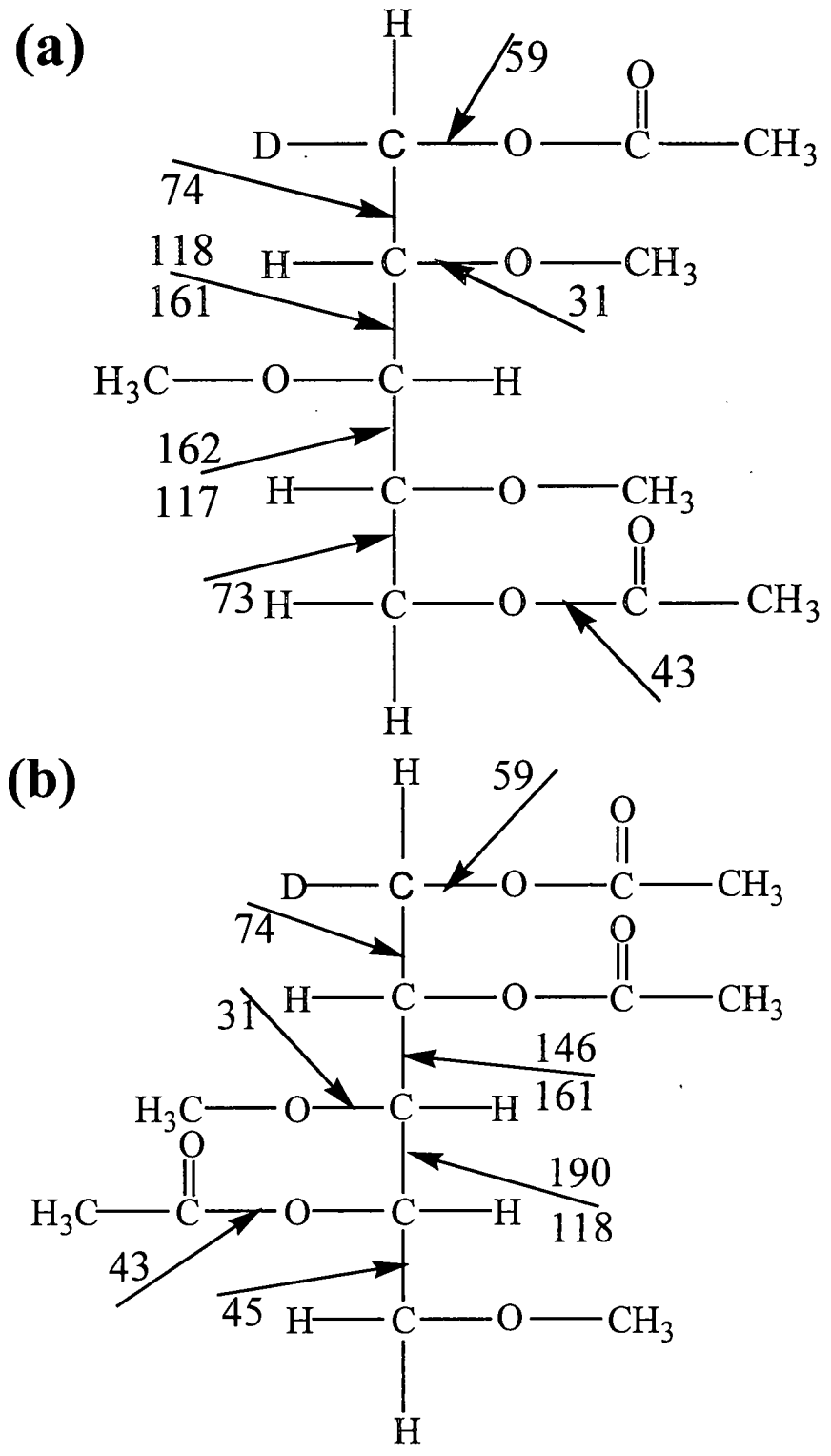


Fig. 3.36: Formulae of fragments of compound B after MS from figure 3.35: (a) peak 73 assuming pentitol is D-[1-^{2}H]xylitol and (b) peak 152 assuming pentitol is L-[1-^{2}H]arabinitol.

Mass No (peak 73)	Identified fragment (total MW = 279)	Mass No (peak 152)	Identified fragment (total MW = 307)
43	$-COCH_3$	43	$-COCH_3$
45	$-CH_2-O-COCH_3$	45	$-CH_2-O-COCH_3$
59	$-O-COCH_3$	88	$-CH_2-O-COCH_3 + -COCH_3$
88	$-CH_2-O-COCH_3 + -COCH_3$	101	117 – MeOH or 161 – HOAc
101	117 – MeOH or 161 – HOAc	102	118 – MeOH or 162 – HOAc
102	118 – MeOH or 162 – HOAc	129	161 - MeOH
117	307 – 190	130	190 - HOAC
161	307 – 146	161	101 + HOAC or 279 - 118
162	117 + 45	162	117 + 45
		190	307 - 117

Table 3.9: M_r of possible fragments after GC-MS of permethylated compound B.

With the help of the molecular weight of possible fragments (Fig. 3.36 and Table 3.9) after GC-MS of permethylated compound B, peak 73 was identified as 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl xylitol and peak 152 as 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methyl arabinitol.

From this result it can be concluded that the Ara_f unit is substituted at position O-2 by a non-reducing Xyl_p unit within compound B.

Taken together, the evidence shows that compound B is a feruloylated disaccharide of L-ararbinose and D-xylose with arabinose as the reducing and xylose as the non-reducing terminus. The xylose residue is attached to *O*-2 of arabinose and is in β-configuration. Ferulate is the sole ester group present in compound B.

3.6 Investigation of compound C
3.6.1 The proposed structure

The proposed structure of compound C connected to its parent polymer is given is figure 3.37.

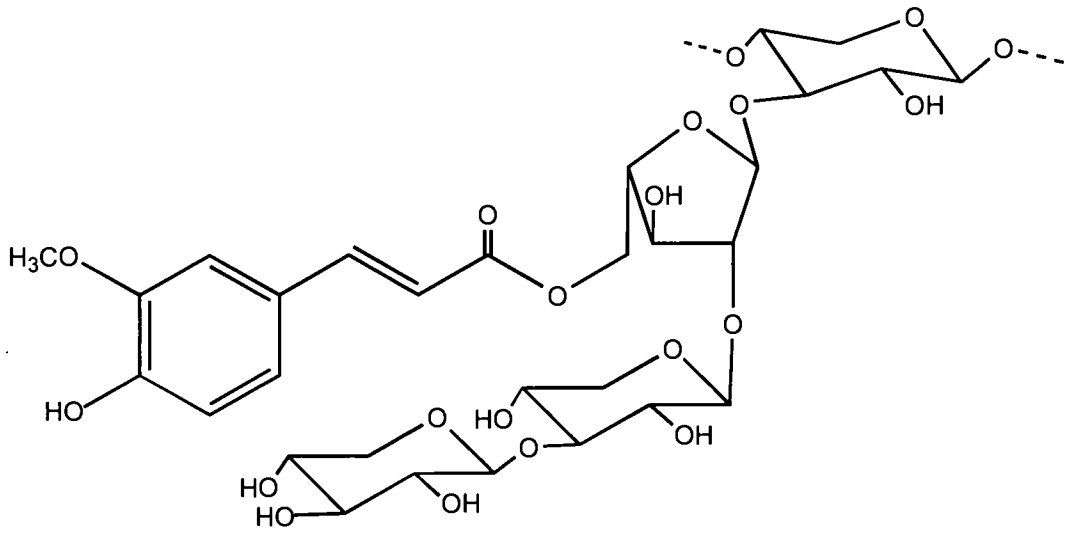


Fig. 3.37: The proposed structure of compound C connected to its parent polymer.

3.6.2 Calculation of the specific radioactivity of [^{3}H]arabinose and [^{3}H]xylose residues in the total AIR

The specific radioactivities served as the basis for the calculation of the arabinose : xylose ratios for compounds C, D, E and for compounds released after Driselase-hydrolysis (§ 3.10).

Severe acid hydrolysis (§ 2.6.1) was conducted on a portion of ^{3}H-labelled AIR. The hydrolysate was subjected to PC in EPW_1 (§ 2.6.1). The arabinose and xylose (§§ 2.6.8 and 3.5.5.1) were eluted and assayed ① for ^{3}H by scintillation counting in TS (§ 2.8.1.2) (Table 3.10) and ② for reducing sugars by the PAHBAH-assay (§ 2.8.2) (Table 3.12) using L-arabinose and D-xylose for standard curves (Fig. 3.38).

Aliquot No.	blank cpm/10 µl	H#	arabinose cpm/10 µl	H#	xylose cpm/10 µl	H#
1	120	62.7	298235	64.6	132112	64.3
2	106	61.6	292253	64.4	132099	64.6
3	100	62.3	284617	64.0	145153	64.5
4	106	61.4	308250	63.4	142898	64.8
5	116	63.2	318096	62.8	136194	64.4
6	103	61.4	327415	63.8	144983	65.6
7	103	61.9	301376	63.5	138725	65.0
8	101	60.8	310376	63.1	141797	63.3
9	101	62.7	314631	62.7	143436	63.0
10	110	61.9	312355	63.1	137292	64.2
$\bar{x}$	106.6	61.9	306700	63.5	139500	64.4
σ_{n-1}	6.77	0.732	12760	0.65	4945	0.76

Table 3.10: Blank, [^{3}H]Ara and [^{3}H]Xyl were assayed for radioactivity. Ten 10-µl/ml aliquots of blank (§ 2.6.8) were taken, H# = quench number.

Parameter	arabinose	xylose
$\bar{x}$ sugar (cpm/10 µl)	306700	139500
$\bar{x}$ blank(cpm/10 µl)	106	106
corrected (cpm/10 µl)	306600	139400
% efficiency from ^{3}H-Quench curve	45.7	45.6
dpm/10µl	670900	305700
kBq/10µl	40250	18340
total MBq in 1 ml	4025	1834

Table 3.11: Calculation of total radioactivity per 1 ml eluate.

The total radioactivity was 4025 MBq for L-arabinose and 1834 MBq for D-xylose (Table 3.11). The specific radioactivities of [^{3}H]Ara and [^{3}H]Xyl in the total AIR were 252 MBq/mg and 99.7 MBq/mg, respectively (Table 3.13).

Repetition No	absorbance at 410 nm		
	blank	arabinose (1 : 50)	xylose (1 : 50)
1	0.037	0.174	0.124
2	0.036	0.194	0.142
3	0.045	0.210	0.180
4	0.038	0.202	0.190
5	0.035	0.196	0.174
6	0.041	0.246	0.170
7	0.047	0.210	0.170
8	0.039	0.224	0.172
9	0.038	0.186	0.178
10	0.043	0.226	0.168
$\overline{x}$	0.040	0.207	0.167
σ_{n-1}	0.004	0.0211	0.0194

Table 3.12: PAHBAH-assay. Ten 25-µl aliquots of blank, [^{3}H]Ara and [^{3}H]Xyl out of 1 ml eluate were assayed for reducing sugar.

Parameter	arabinose	xylose
A_{410} from PAHBAH-assay	0.207	0.167
A_{410} of blank	0.040	0.040
Δ (A_{410})	0.167	0.127
Pentose from standard curves in µg/10µl (Fig. 3.38)	2.15	2.20
total mg in 1 ml eluate	17.2	17.6
specific activity in total AIR (MBq/mg)	252	99.7

Table 3.13: Calculation of the specific radioactivity of [^{3}H]arabinose and [^{3}H]xylose.

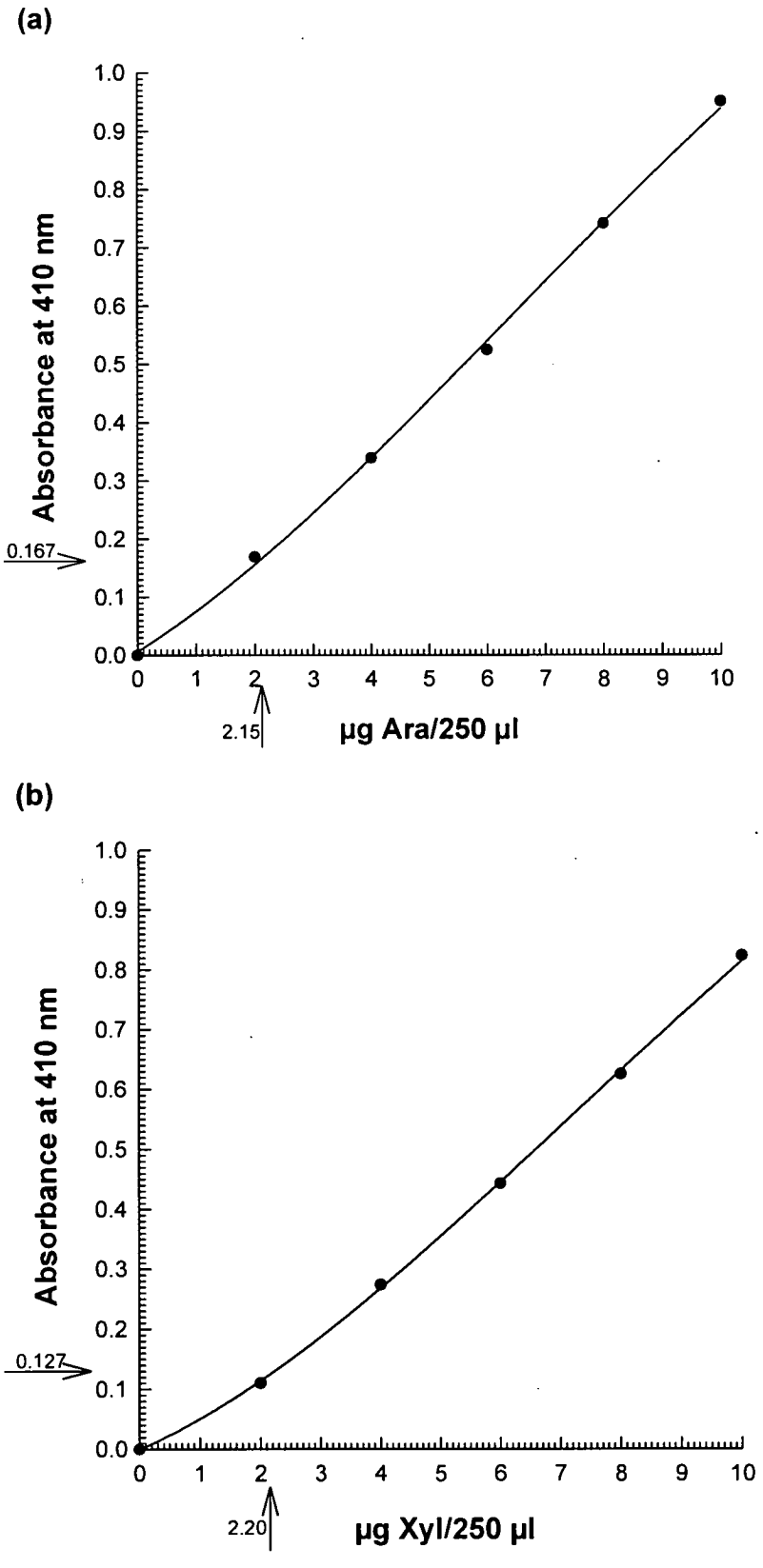


Fig. 3.38: Standard curves for PAHBAH assay of L-arabinose (a) and D-xylose (b) previously dried in a thermostat vacuum oven over phosphorus pentoxide for 9 hours. Each point is an average of 4 assays.

3.6.3 Monosaccharide composition

To determine the pentose composition of ^{3}H-labelled compound C, a portion was subjected to severe acid hydrolysis (§ 2.6.1) and chromatographed in EPW_1 (§ 2.5.1). Figure 3.39 shows two radioactive products: [^{3}H]arabinose and [^{3}H]xylose in the ratio ≈1.31 : 1.0 (^{3}H-basis), which corresponds to a molar ratio of 0.519 : 1.0 by taking into account the specific activity (Table 3.13). Assaying in TS (§ 2.7.1.2) gave ratios of ≈1.24 : 1.0 (^{3}H-basis) and 0.492 : 1.0 (molar basis), suggesting a trisaccharide (Ara, Xyl_2).

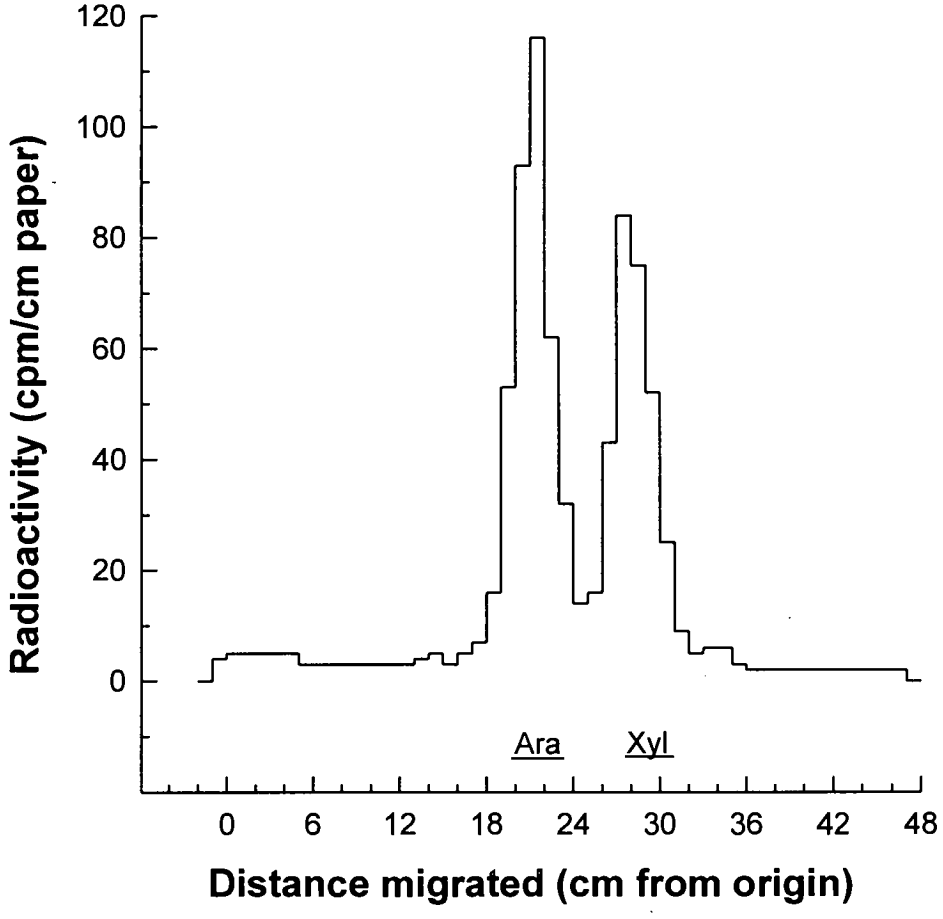


Fig. 3.39: PC in EPW_1 (§ 2.5.1) of severe acid hydrolysis (§ 2.5.1) products of ^{3}H-labelled compound C_S. Ara and Xyl served as external and internal markers.

3.6.4 Gel-permeation chromatography on Bio-Gel P-2 of compound C_S

To establish that compound C_S is a trisaccharide was further tested by gel-permeation chromatography on Bio-Gel P-2 (§ 2.5.5). By reference to internal marker sugars compound C_S was confirmed to be a trisaccharide (Fig. 3.40).

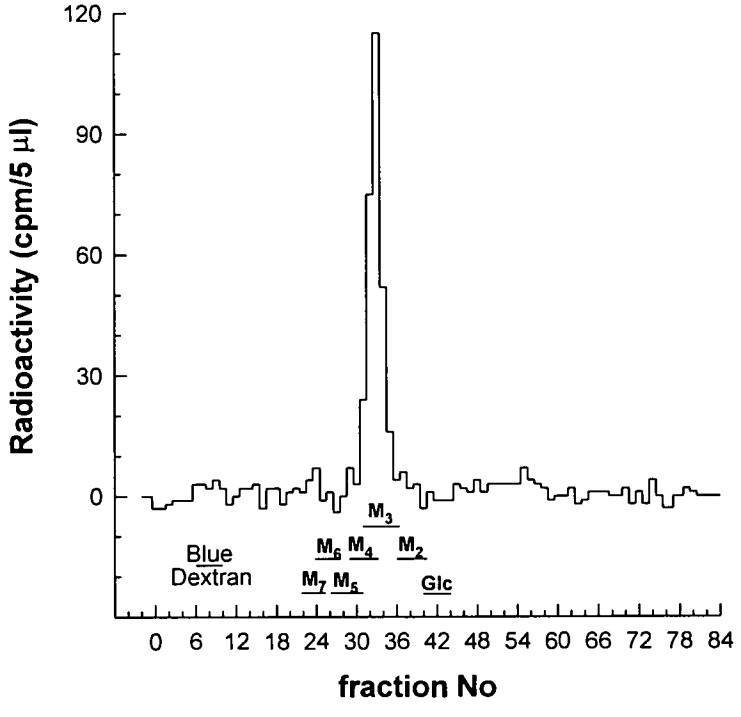


Fig. 3.40: Behaviour of ^{3}H-labelled compound C_S on Bio-Gel P-2 (§ 2.5.5). Blue Dextran, glucose, maltose ... and maltoheptaose were used as internal markers.

To determine the nature of the reducing terminus, the trisaccharide C_S was reduced (§ 2.6.6) and subjected to severe acid hydrolysis (§ 2.6.1). Arabinose and arabinitol could be successfully separated from xylose (Fig. 3.41) by PC in EPW_1 (§ 2.5.1). The reduced and hydrolysed trisaccharide (= C_S-ol) yielded [^{3}H]arabinose/[^{3}H]arabinitol and [^{3}H]xylose in the ratio ≈1.42 : 1.0 (^{3}H-basis; Fig. 3.41) which corresponds to a molar ratio of 0.562 : 1.0 by taking into account the specific activity (Table 3.13). This ratio indicates a trisaccharide with arabinose as reducing terminus.

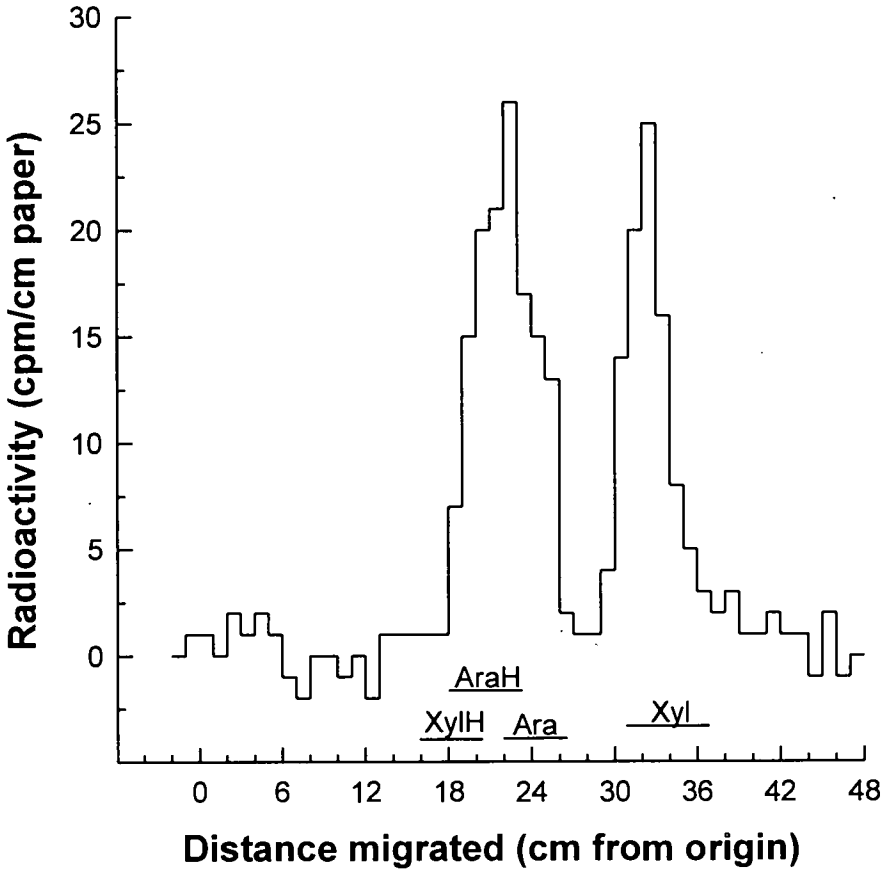


Fig. 3.41: PC in EPW_1 (§ 2.5.1) of severe acid hydrolysis products (§ 2.5.1) of reduced trisaccharide C_S. Ara, AraH, Xyl and XylH were used as external markers.

3.6.5 Hydrolysis with Driselase

3.6.5.1 Stability of compound B in Driselase

It was found that compound B_S is resistant to hydrolysis by Driselase (see § 4.4.1). Here, the Driselase-hydrolysis of ^3H-labelled compound B is quantified (Fig. 3.42).

Compound B in figure 3.42 (a) accounted for 65.9% of the total ^3H in the sample. After hydrolysis with Driselase, 58.0% of the ^3H was present as B (Fig. 3.42 (b)). This result means that less than 12% of compound B was hydrolysed.

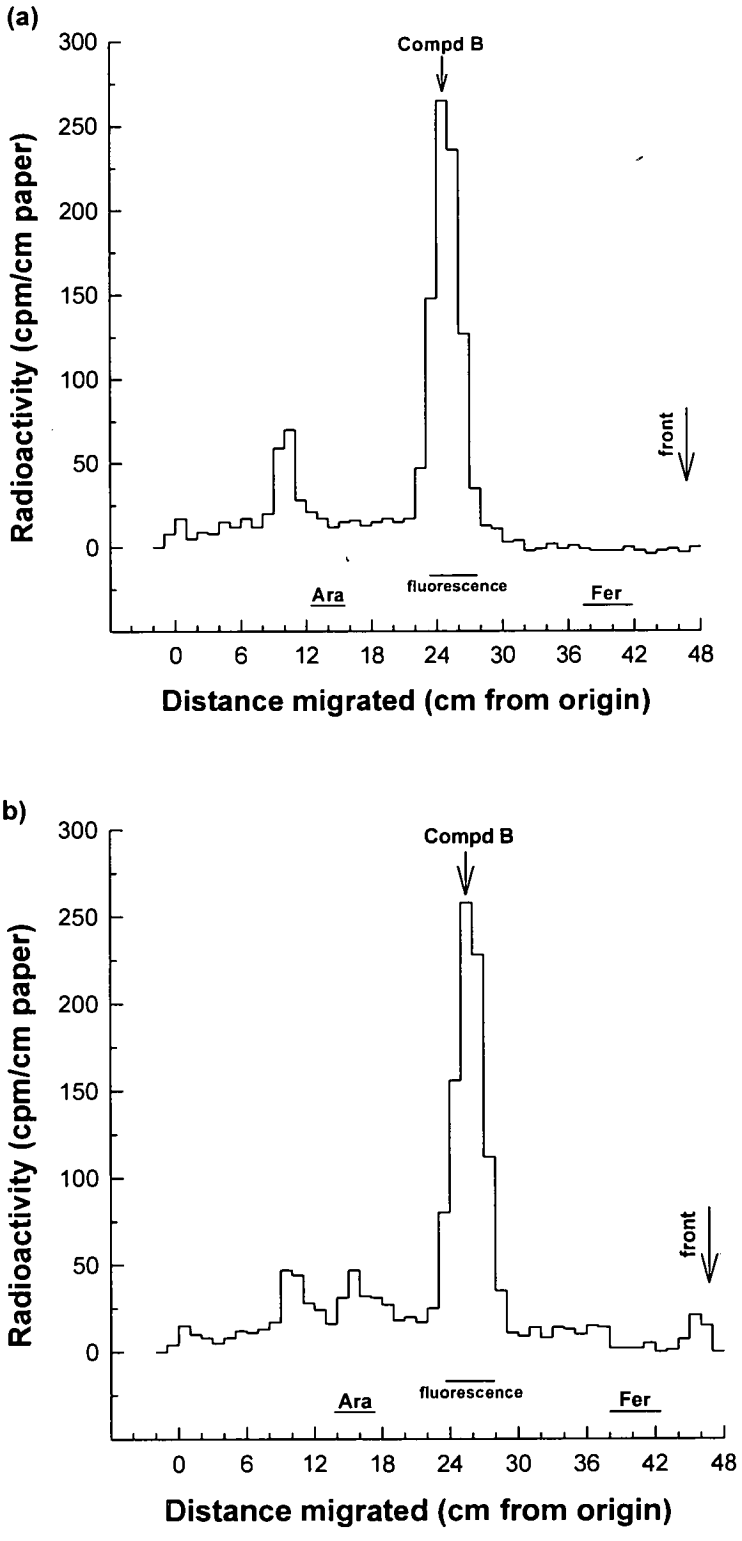


Fig. 3.42: PC in BAW (§ 2.5.1) of Driselase hydrolysis products of ^{3}H-labelled compound B. (a) Compd B with buffer alone and (b) compd B with Driselase. Ara and Fer were used as external markers and non-labelled compound B as internal marker. "Compd B" shows the approximate position expected of this compound (Table 3.1).

3.6.5.2 Driselase-hydrolysis of compound C

Treatment of ^{3}H-labelled compound C with Driselase may show whether compound B is part of this compound or not. The hydrolysis was performed over a time course (§ 2.6.3.1.2) (Fig. 3.43).

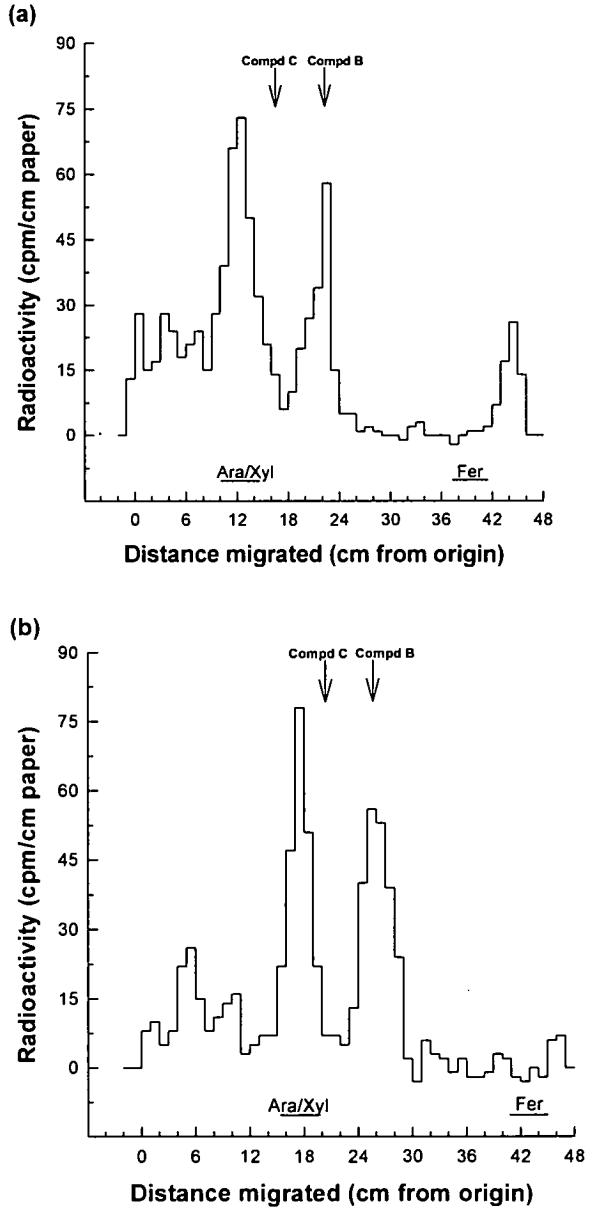


Fig. 3.43: PC in BAW (§ 2.5.1) of Driselase hydrolysis products of ^{3}H-labelled compound C. (a) Hydrolysis-products after 48 h and (b) hydrolysis-products after 96h. Ara, Xyl and Fer served as external markers. "Compd B" and "Compd C" show the approximate position expected of compounds B and C, respectively (Table 3.1) (see also Fig. 3.47 (a)).

3.6.5.3 Identification of compound B as component of C

The arrows in figure 3.43 indicate the R_{Ara}-values (Table 3.1) of compound B and C, respectively. It is clear that compound C had been partially hydrolysed to a free pentose (Ara or Xyl) plus a product that approximately co-chromatographed with B. This "compound B" (24 to 30 cm from the origin of figure 3.43 (b)) was eluted (§ 2.6.4) and subjected again to PC in BAW with non-labelled compound B as internal marker. Figure 3.44 shows that "compound B" (Fig. 3.43 (b)) co-migrated exactly with authentic compound B as an internal marker.

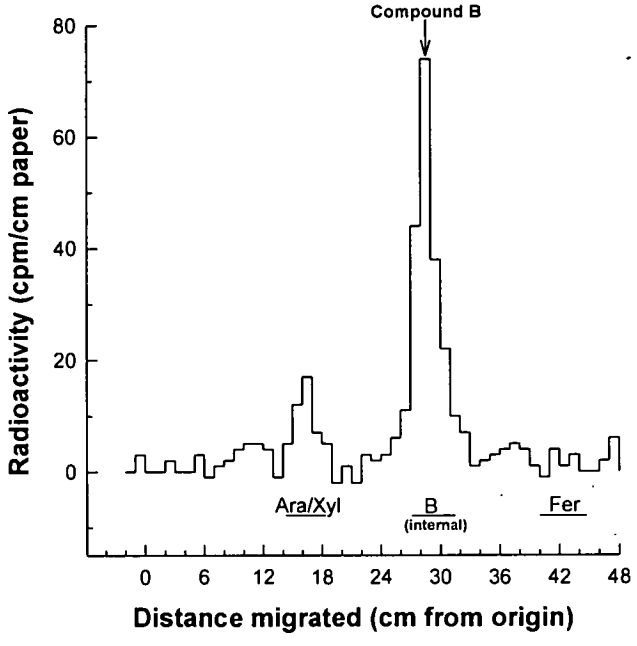


Fig. 3.44: Re-chromatography in BAW (§ 2.5.1) of "compound B" (24 to 30 cm from the origin of figure 3.43 (b)). Ara, Xyl and Fer were used as external and non-radioactive compound B as an internal marker. "Compd B" shows its approximate position (Table 3.1).

Driselase-hydrolysis of ^{3}H-labelled compound C thus revealed that the compound is made of compound B plus an additional ^{3}H-pentose. Although arabinose and xylose can not be fully separated in BAW, the additional pentose is likely to be xylose since the ratio of arabinose to xylose in compound C is ~1 : 2.

3.6.6 Smith degradation

In order to identify the glycosidic linkage of the additional xylose residue to compound B in compound C, Smith degradation (§ 2.6.7) was performed with a portion of ^{3}H-labelled compound C_S-ol. The products were separated by PC in EPW_1 (Fig. 3.45).

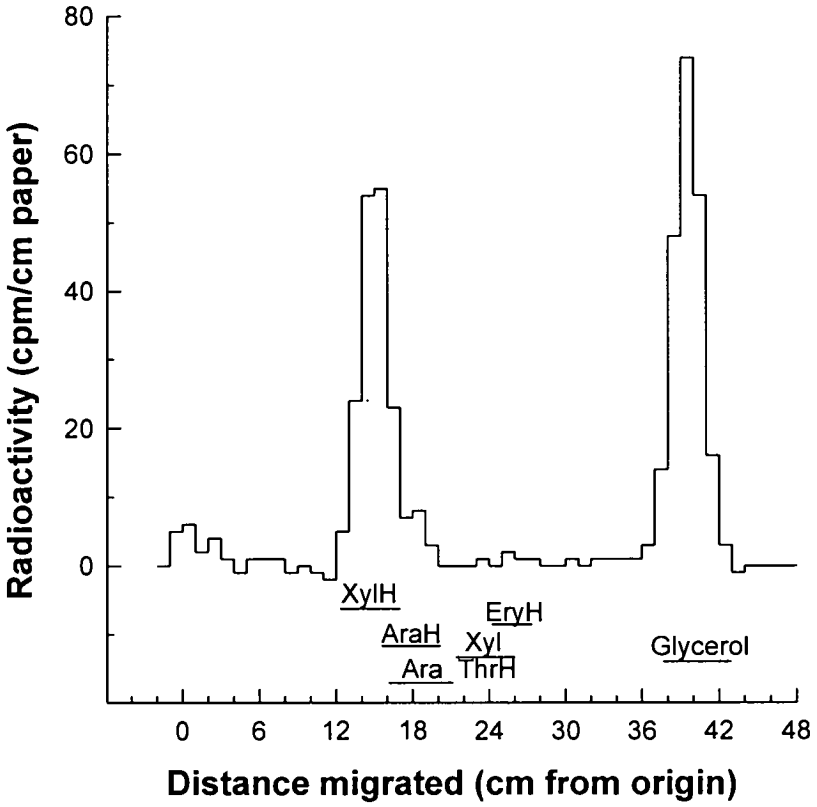


Fig. 3.45: PC in EPW_1 of products of ^{3}H-labelled compound C_S-ol after $NaIO_4$-oxidation, TFA hydrolysis and $NaBH_4$-reduction. Ara, AraH, Xyl, XylH, EryH, ThrH and glycerol were external markers.

Figure 3.45 shows the presence of [^{3}H]xylitol (R_{Ara}-value of 0.84) and [^{3}H]glycerol (R_{Ara}-value of 2.05). The [^{3}H]glycerol would arise from the 2-linked L-[1-^{3}H]arabinitol moiety. Since [^{3}H]xylitol was also released, a (1→3)-linkage of the additional xylose to the xylose residue of compound B can be suggested (Fig. 3.37).

3.6.7 Kinetics of alkaline hydrolysis

3.6.7.1 Determination of the half-life

To examine the alkali lability of the *O*-feruloyl ester bond within compd C, alkaline hydrolysis of ^{3}H-labelled compd C was conducted at pH ~13.3 and 25°C (§ 2.6.2) and the products were examined chromatographically. The half-life was estimated at ~5 min (Fig. 3.46). Figure 3.47 shows chromatograms of the material sampled during the hydrolysis.

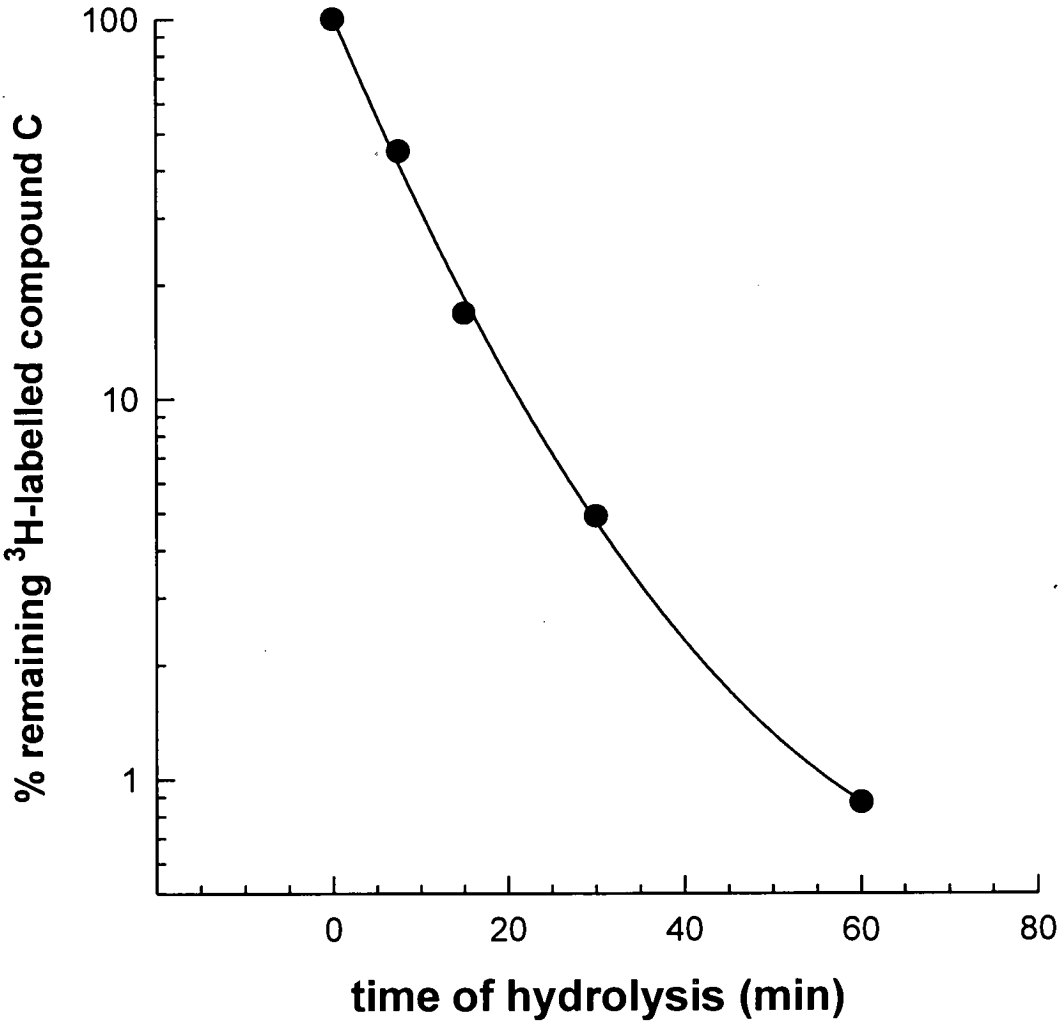


Fig. 3.46: Alkaline hydrolysis (§ 2.6.2) of ^{3}H-labelled compound C over a time course.

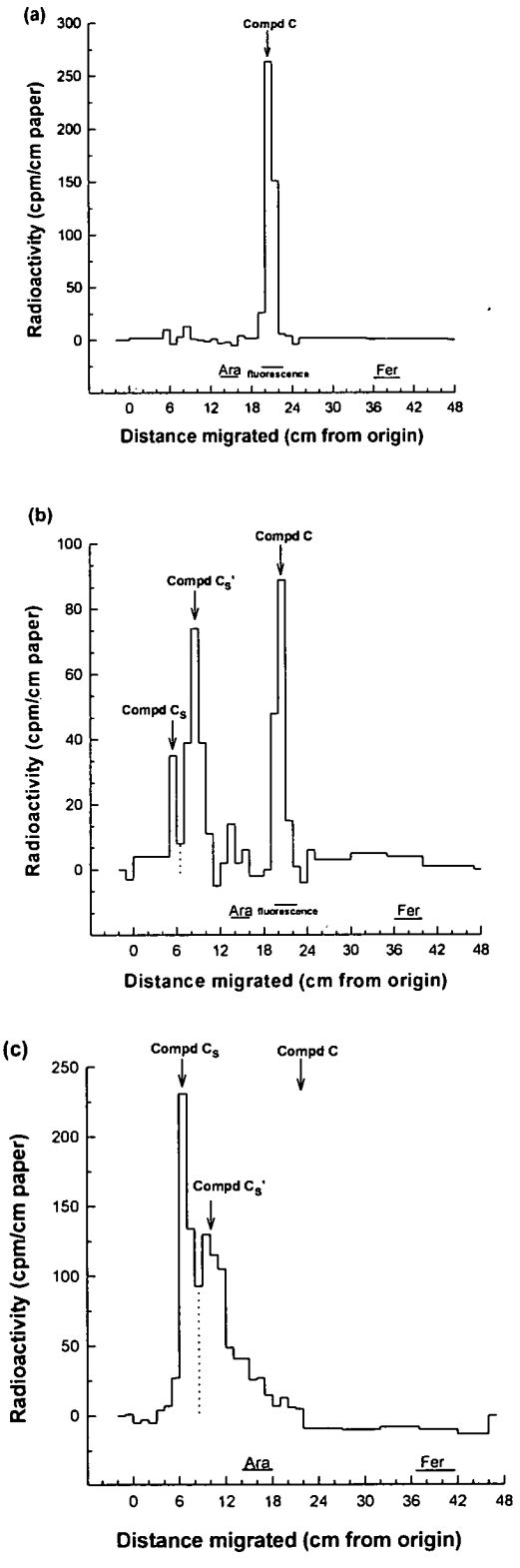


Fig. 3.47: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of ^{3}H-labelled compound C formed after (a) 0 min, (b) 7.5 min and (c) 60 min. Ara and Fer served as external markers. "Compd C" and "Compd C_S' " show the approximate position of these compounds (Table 3.1).

3.6.7.2 Test for multiple esters

To test for multiple esters in compound C, partial saponification at pH ~13.3 and 25°C of (*feruloyl*-^{14}C)-labelled compound C was performed (Fig. 3.48).

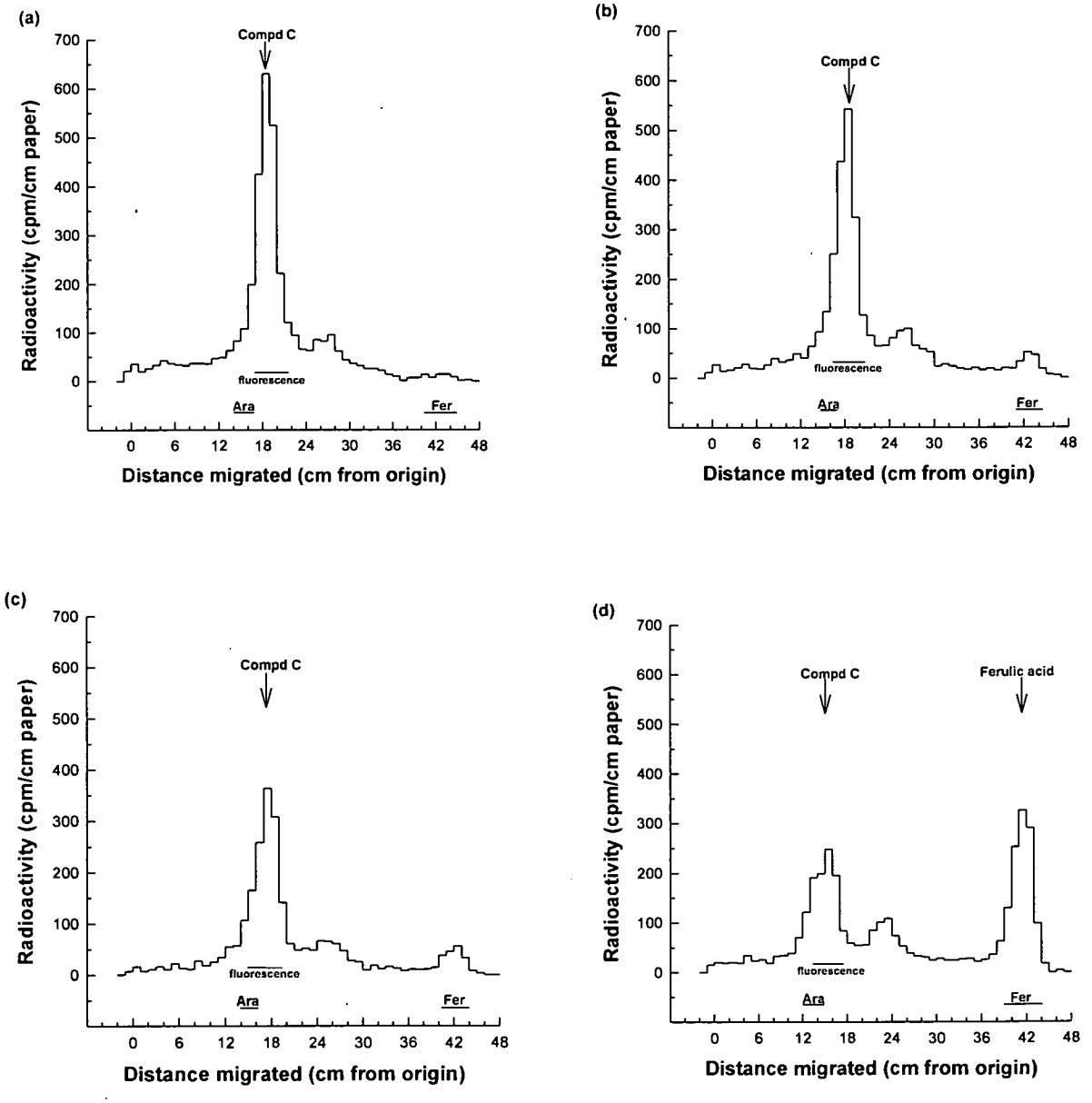


Fig. 3.48: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of ^{14}C-labelled compound C formed after (a) 0 min, (b) 0.5 min, (c) 1 min and (d) 8 min. Ara and Fer were used as external markers. "Compd C" shows the approximate position of this compound (Table 3.1).

Figure 3.48 shows that no intermediate product was formed (a product whit lower R_{Ara}-value than the original compound). The peak with a higher R_{Ara}-value (R_{Ara} = 2.00) than original compound C (R_{Ara} = 1.36) is likely to be an impurity (it is already present in the 0-min sample). Therefore it can be concluded that ferulate is the sole ester-linked group in compound C.

From these data, the half-life of compound C can be calculated to ~8 min (Fig. 3.49).

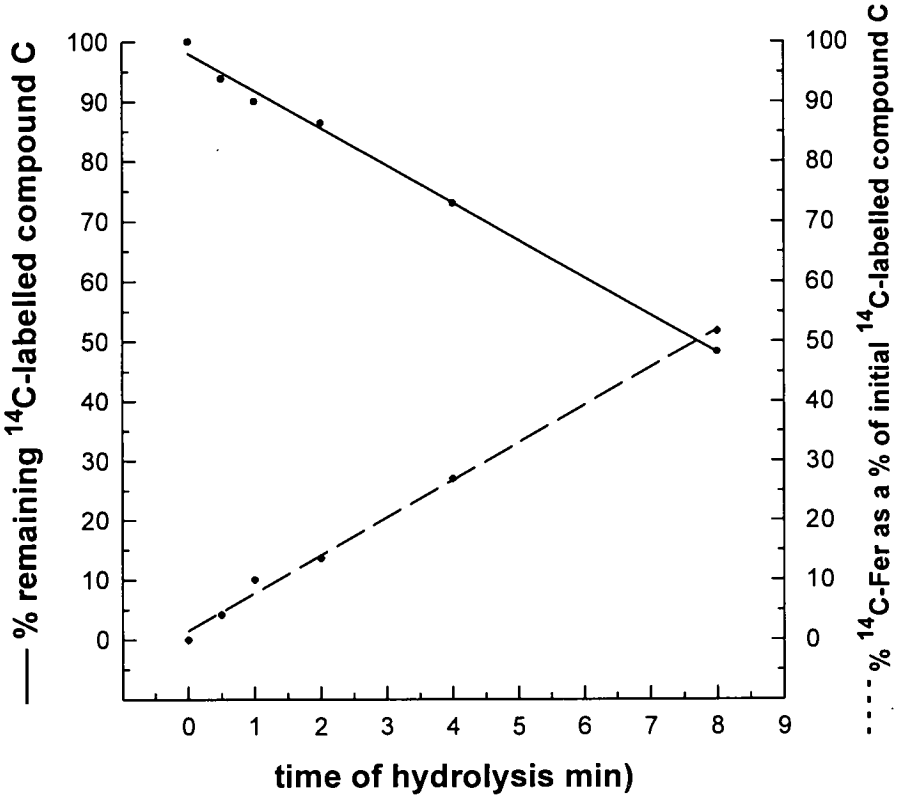


Fig. 3.49: Short-term kinetics of alkaline hydrolysis (§ 2.6.2) of ^{14}C-labelled compound C.

Taken together, it is believed that compound C is a feruloylated trisaccharide consisting of compound B with an additional xylose residue. The additional xylose is attached to *O*-3 of the xylose of compound B. Ferulate is the sole ester group present in compound C.

3.7 Investigation of compound D

3.7.1. Monosaccharide composition

To determine the sugar composition of compound D, a portion (see § 3.1.1 for purification) of the (*pentosyl*-^{3}H)-labelled compd was subjected to severe acid hydrolysis (§ 2.6.1) and chromatographed in EPW_1 (§ 2.5.1). Figure 3.50 shows two main radioactive products: [^{3}H]arabinose and [^{3}H]xylose in the ratio ≈1.20 : 1.0 (^{3}H -basis) which corresponds to a molar ratio of 0.475 : 1.0 by taking into account the specific activity (Table 3.13), suggesting a tri- or hexasaccharide (Ara, Xyl_2).

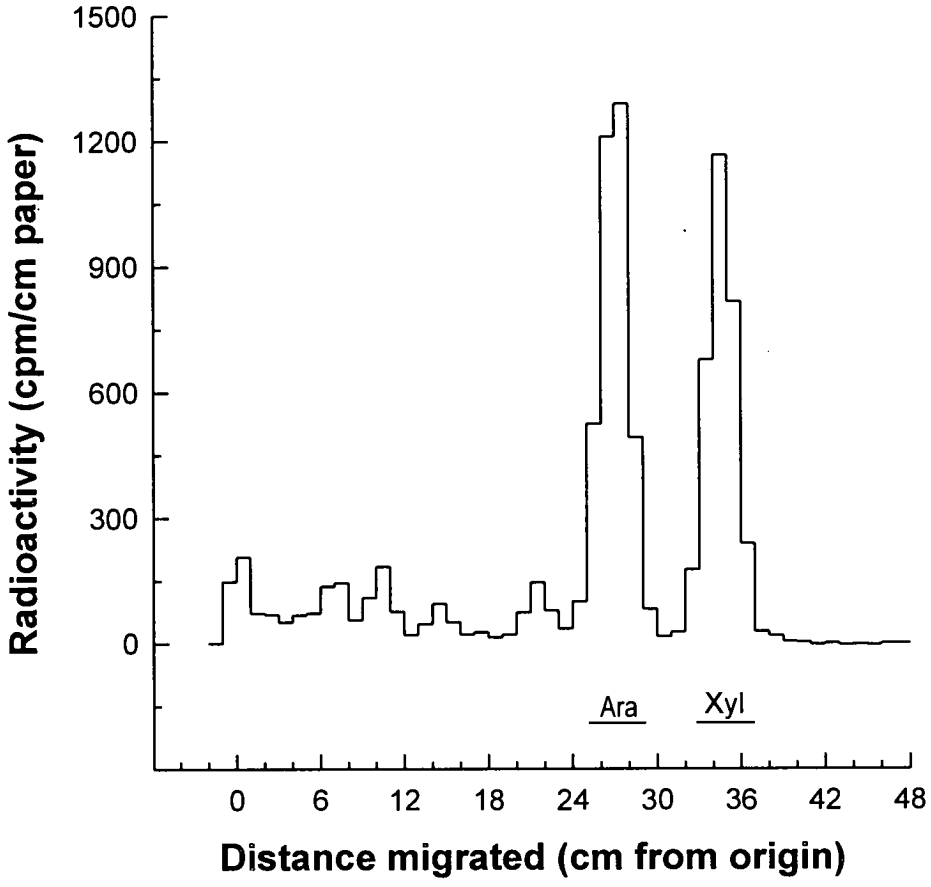


Fig. 3.50: PC in EPW_1 (§ 2.5.1) of severe acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compound D_S. Ara and Xyl served as external and internal markers.

3.7.2 Gel-permeation chromatography on Bio-Gel P-2 of compound D_S

The DP of ^{3}H-labelled compound D_S was tested by gel-permeation chromatography on Bio-Gel P-2 (§ 2.5.5). By reference to internal marker sugars compound D_S was identified as a pentasaccharide or hexasaccharide (Fig. 3.51).

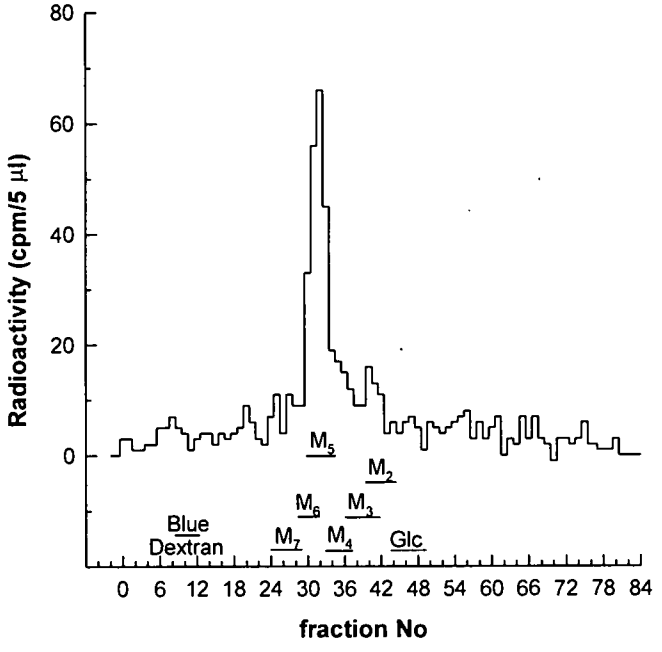


Fig. 3.51: Behaviour of ^{3}H-labelled compound D_S on Bio-Gel P-2 (§ 2.5.5). Blue Dextran, glucose, maltose ... maltoheptaose were used as internal markers.

3.7.3 Hydrolysis with Driselase

3.7.3.1 Hydrolysis of compound D

Treatment of ^{3}H-labelled compound D with Driselase will answer the question as to whether compound B is part of this compound or not. The hydrolysis was performed over a time course (§ 2.6.3.1.2). The results showed (Fig. 3.52) that compound D was hydrolysed by Driselase into smaller fragments.

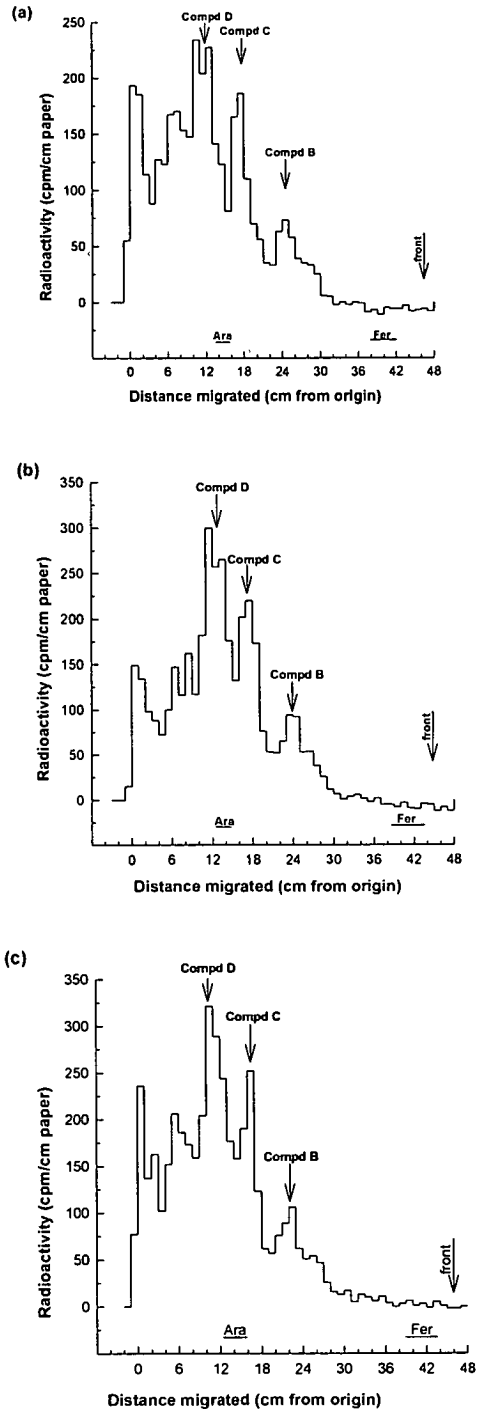


Fig. 3.52: PC in BAW (§ 2.5.1) of Driselase hydrolysed (§ 2.6.3.1.2) products of ^{3}H-labelled compound D. (a) Hydrolysis-products after 48 h, (b) after 96h and (c) after 144 h. Ara and Fer served as external markers. "Compd B", "Compd C" and "Compd D" show the approximate position of these compounds (Table 3.1); for untreated ^{3}H-labelled compound D see also figure 3.57 (a).

3.7.3.2 Identification of compounds B and C as components of D

It is clear that ^{3}H-labelled compound D has been broken down by Driselase. "Compound B" (21 to 30 cm from the origin in figure 3.52 (c)) and "compound C" (16 to 19 cm from the origin) were eluted (§ 2.6.4) and subjected again to PC in BAW (§ 2.5.1) with non-labelled compounds B and C as internal markers. Figure 3.53 shows that the ^{3}H-products obtained by Driselase-digestion of ^{3}H-labelled compound D co-migrated exactly with compound B and compound C.

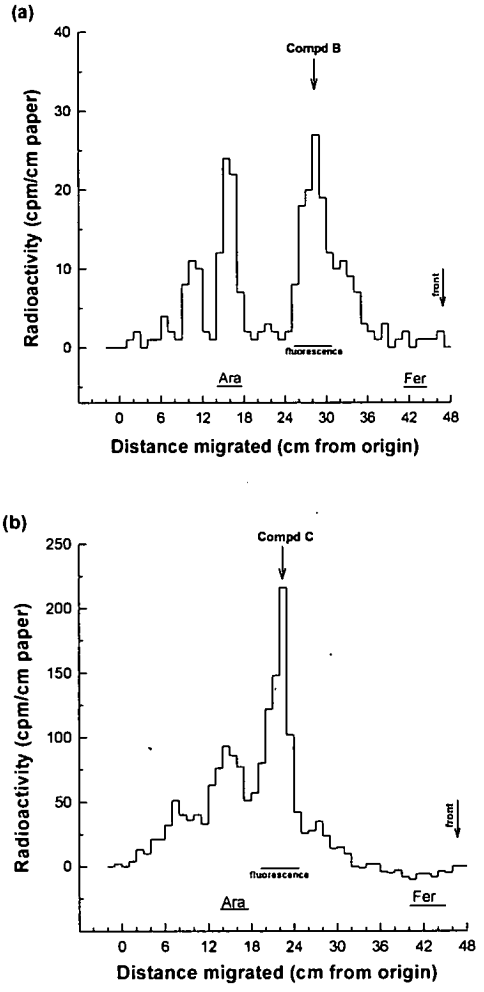


Fig. 3.53: PC in BAW (§ 2.5.1) of (a) "compound B" from figure 3.52 (c) and (b) "compound C" from figure 3.52 (c). Ara and Fer were used as external and non-labelled compound B and compound C ("fluorescence") as internal markers. "Compd B" and "Compd C" show the approximate position of these compounds (Table 3.1) (see also figures 3.17 (a) and 3.47 (a)).

Since compound D approximately co-chromatographs with free pentoses in BAW, it is not obvious whether the peak of ^3H at "compound D" in figure 3.52 (c) is Ara, Xyl, remaining D, or a mixture. To see whether ^3H-labelled compound D had been broken down completely, compound D (10 to 14 cm from the origin from figure 3.52 (c)) was eluted (§ 2.6.4) and purified on RPC (§ 2.6.5). The MeOH-fraction was subjected to PC in BAW (§ 2.5.1) with non-labelled compound D as internal marker (Fig. 3.54 (a)) and the H_2O-fraction to PC in EPW_1 (§ 2.5.1) (Fig. 3.54 (b).

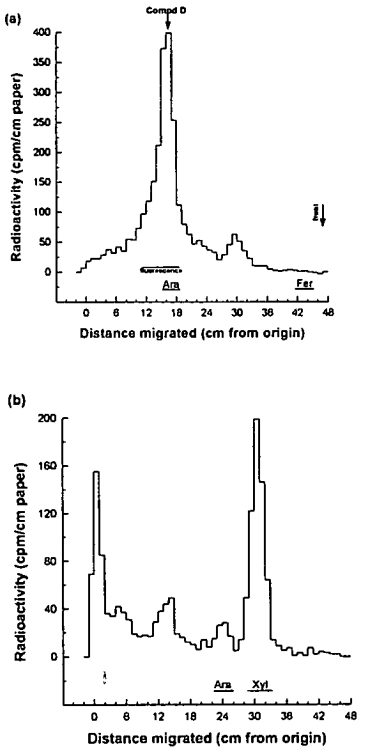


Fig. 3.54: PC of RPC fractions of compound D (10 to 14 cm from the origin in figure 3.52 (c)): products of the MeOH-fraction (a) were separated in BAW (§ 2.5.1) and products of the H_2O-fraction (b) in EPW_1. Ara, Xyl and Fer were used as external and non-labelled compd D as an internal marker. "Compd D" shows the approximate position of this compound (Table 3.1).

The results reveal that Driselase-hydrolysis did not completely break down ^3H-labelled compound D to compound B and additional sugars within 144 h. Remaining compound D (Fig. 3.54 (a)) and some compound C could be detected as well as xylose and a trace of arabinose (Fig. 3.54 (b)).

3.7.4 Kinetics of alkaline hydrolysis

3.7.4.1 Determination of the half-life

To examine the alkali lability of compound D, alkaline hydrolysis was conducted with a portion of ^{3}H-labelled compound at pH ~13.3 and 25°C (§ 2.6.2) and the product(s) examined chromatographically. Figure 3.55 shows compound D after 0 min and 30 min saponification.

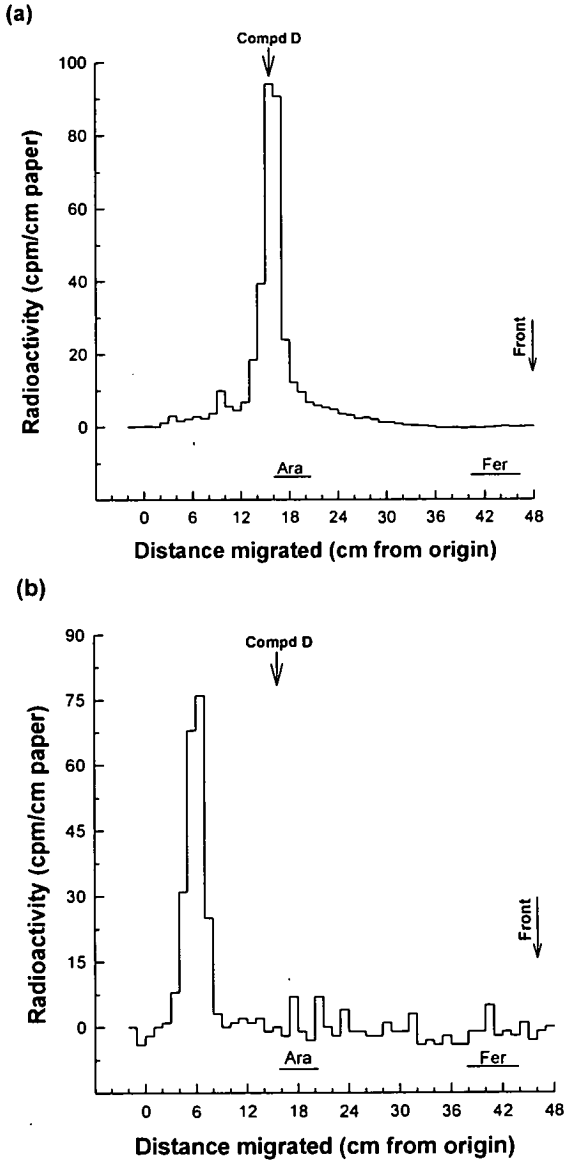


Fig. 3.55: PC in BEW (§ 2.5.1) of alkaline hydrolysis (§ 2.6.2) products of ^{3}H-labelled compound D formed after (a) 0 min and (b) 30 min. Ara and Fer served as external markers. "Compd D" shows the approximate position of this compound (Table 3.1).

Since complete hydrolysis took place within the first 30 min (Fig. 3.55), alkaline hydrolysis of a portion of ^{14}C-labelled compound D was carried out at pH ~13.3 and 25°C (§ 2.6.2) over a shorter time course. Figure 3.56 indicates a half-life of ~4 min and reveals that the yield of ^{14}C-ferulate after 4 min was less than the consumption of ^{14}C-labelled compound D, suggesting multiple esters.

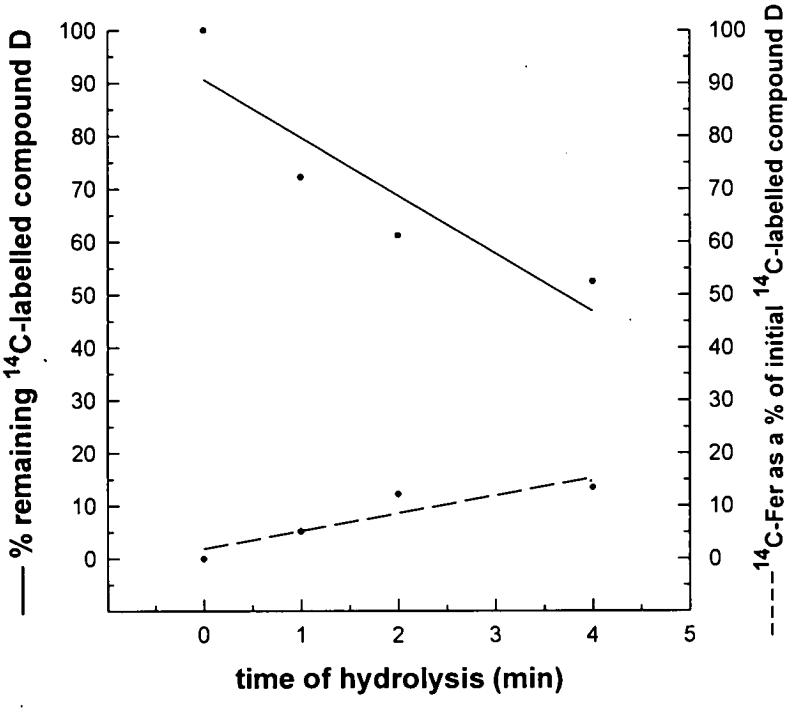


Fig. 3.56: Short-term kinetics of alkaline hydrolysis (§ 2.6.2) of ^{14}C-labelled compound D.

3.7.4.2 Test for multiple esters

To test for multiple esters in compound D, partial saponification of (*ferulate*-^{14}C)-labelled compound D (§ 2.6.10) was carried out. Figure 3.57 shows that at least one intermediate product was formed: compound D', a product with a lower R_{Ara}-value than the original compound. It can be suggested from this that ferulate is not the sole ester-linked group in compound D.

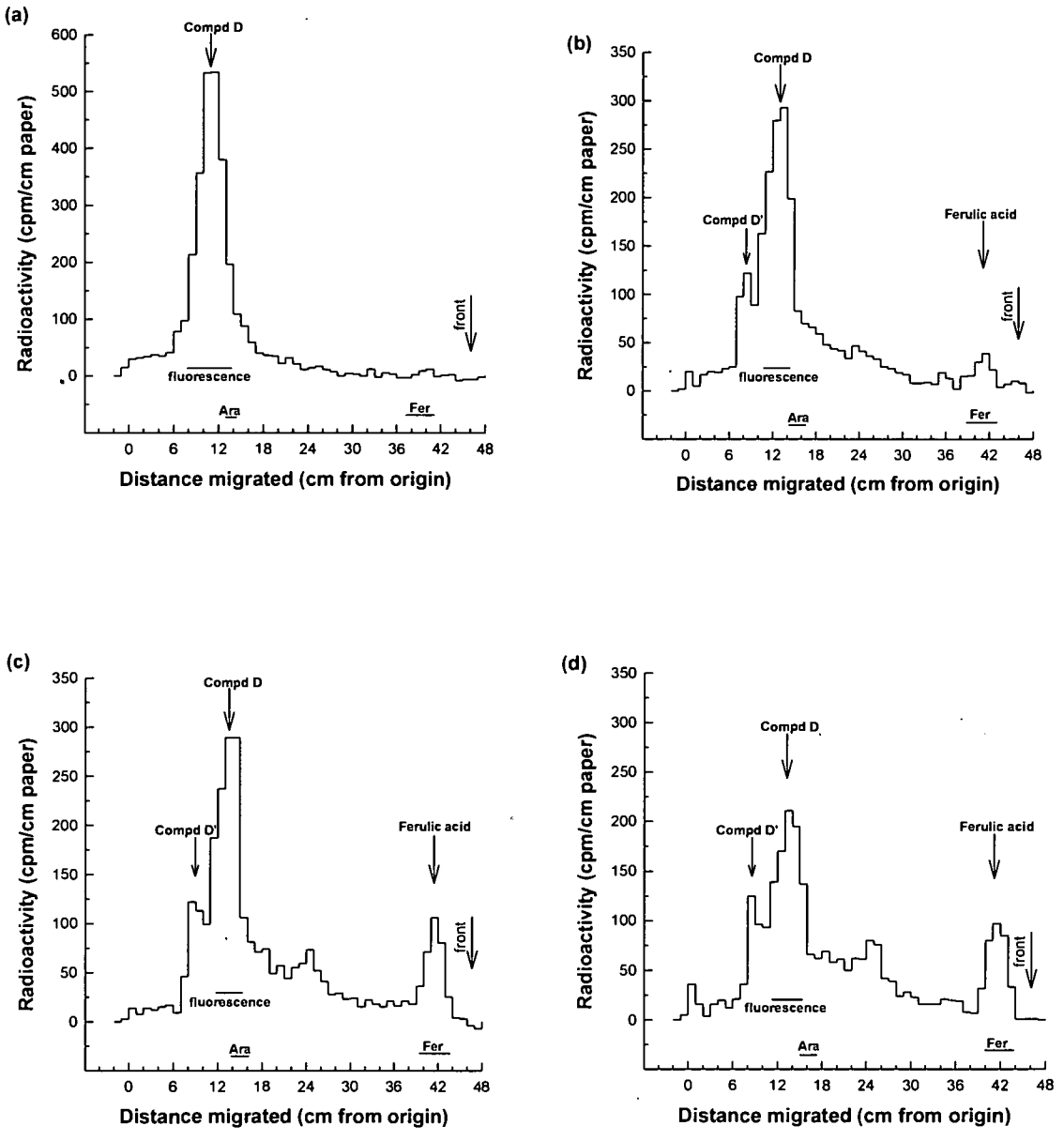


Fig. 3.57: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.6.2) products formed from ^{14}C-labelled compound D after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min. Ara and Fer were used as external markers. "Compd D" and "Compd D' " show the approximate positions of these compounds (Table 3.1). The zero time control sample (a) received the same NaOH and HOAc as samples (b) to (d), but the HOAc was added first. All samples were enriched in phenolic-containing material by reverse-phase column chromatography (§ 2.6.5).

Compound D' (material from 8 cm to 10 cm from the origin in figure 3.57 (c)) was subjected to complete saponification (§ 2.6.2), which yielded ferulic acid and other products (Fig. 3.58).

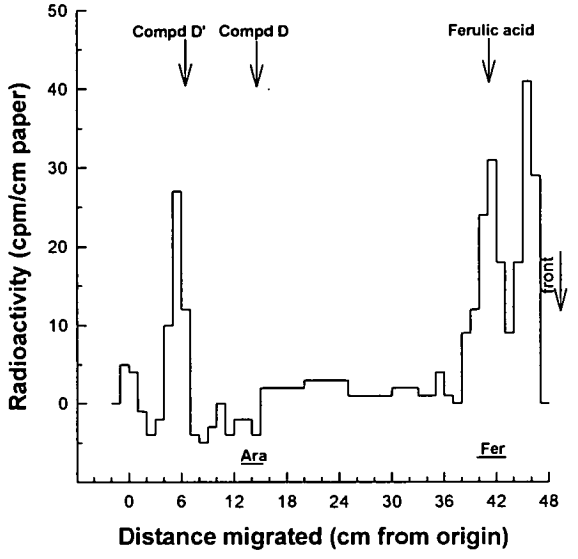


Fig. 3.58: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.6.2) products formed from compound D' after 1 h. Ara and Fer were used as external and Fer in addition as internal marker. "Compd D" and "compd D' " indicate the approximate position of these compounds (Table 3.1).

3.7.5 Identification of the additional ester group

To test whether compound D contains an *O*-acetyl group in addition to *O*-ferulate, a suspension culture of fescue was fed with [^{14}C]acetate over a time course designed to monitor incorporation (§ 2.6.10). AIR prepared from the material at each time point was hydrolysed with mild acid (§ 2.6.1) and a portion of the supernatant assayed for radioactivity. Figure 3.59 shows that the highest yield of incorporated [^{14}C]acetate could be obtained after 32 hours.

In the light of the results of figure 3.59, a new suspension culture of fescue was incubated with [^{14}C]acetate for 32 hours (§ 2.6.10) and the AIR was prepared and treated with mild acid (§ 2.6.1). The hydrolysate was separated by PC in BAW (§ 2.5.1) (Fig. 3.60).

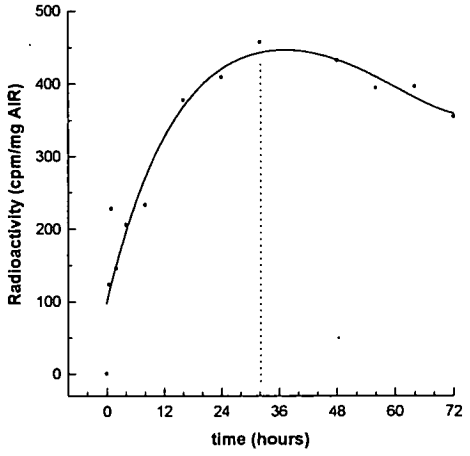


Fig. 3.59: Incorporation of [^{14}C]acetate into AIR of suspension culture of fescue. AIR was prepared (§ 2.6.10) and the hydrolysate (10%) after treatment with mild acid was assayed for radioactivity (§ 2.7.1.2).

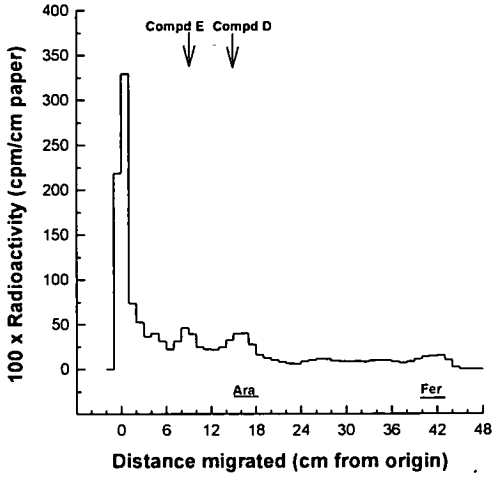


Fig. 3.60: PC in BAW (§ 2.5.1) of mild acid hydrolysate of AIR from [^{14}C]acetate-fed *Festuca* cells. Ara and Fer were used as external markers. "Compd D" and "Compd E" show the approximate positions of these compounds (Table 3.1).

The results of figure 3.60 show that [^{14}C]acetate was successfully incorporated into compound D and E (see § 3.8.5) of fescue arabinoxylan.

Taken together, it is believed that compound D is a penta- or hexasaccharide with a backbone of compound B. It could be shown that in addition to ferulate there is another ester-linked group present, probably acetate.

3.8 Investigation of Compound E

3.8.1 Monosaccharide composition

To determine the pentose composition of compound E, a portion (see § 3.1.1 for purification) of the (*pentosyl*-^{3}H)-labelled compound was subjected to severe acid hydrolysis (§ 2.6.1) and chromatographed in EPW_1 (§ 2.5.1). Figure 3.61 shows two main radioactive products: [^{3}H]arabinose and [^{3}H]xylose in the ratio ≈0.83 : 1.0 (^{3}H-basis), which corresponds to a molar ratio of 0.326 : 1.0 by taking into account the specific activity (Table 3.13), suggesting an oligosaccharide containing 3 times as many xylose as arabinose residues.

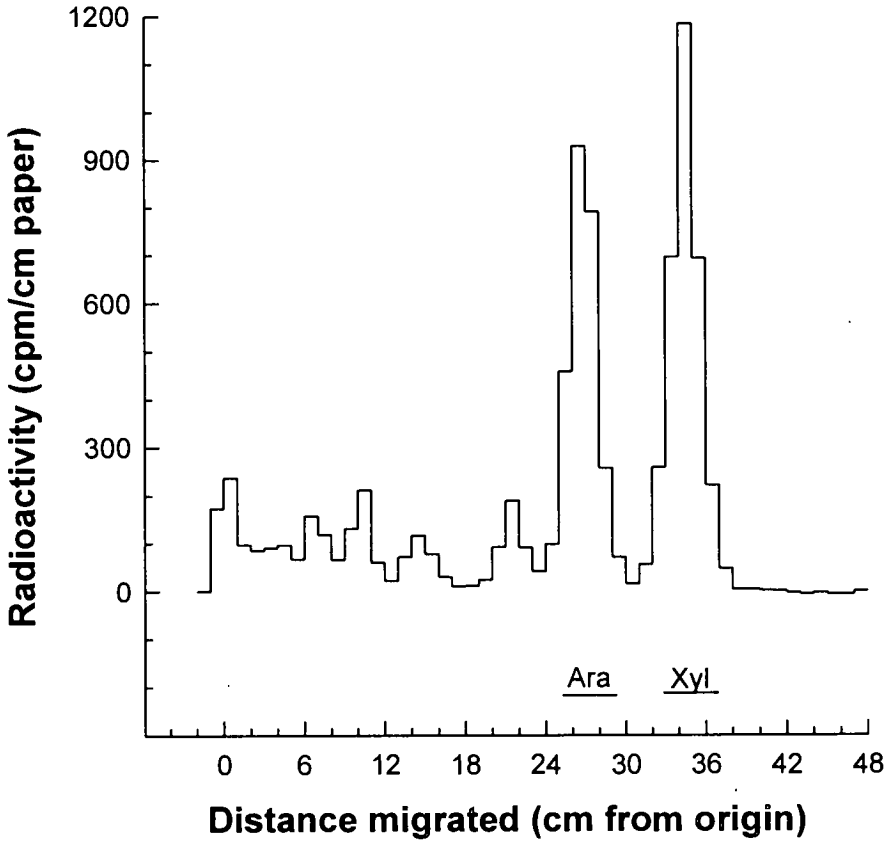


Fig. 3.61: PC in EPW_1 (§ 2.5.1) of severe acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compound E_S. Ara and Xyl served as external and internal markers.

3.8.2 DP of the sugar moiety within compound E

In order to obtain the sugar moiety of compound E, alkaline hydrolysis (§ 2.6.2) was performed. One portion of compound E_S was subjected to PC in EPW_2 (§ 2.5.1) (Fig. 3.62 (a)) and the other portion applied on to a Bio-Gel P-2 column (Fig. 3.62 (b)) (§ 2.5.5). By reference to internal marker sugars (Bio-Gel P-2) and external marker sugars (PC in EPW_2), the major sugar moiety in compound E_S appeared to be of DP ~7.

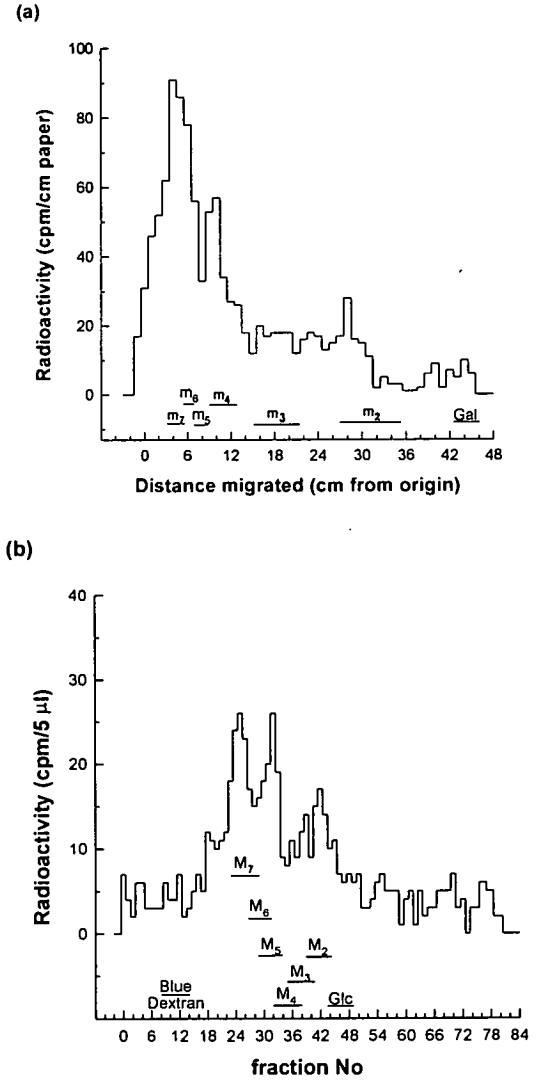


Fig. 3.62: PC in EPW_2 (§ 2.5.1) (a) and (b) gel-permeation chromatography on Bio-Gel P-2 (§ 2.5.5) of compound E_S. Glucose, maltose ... and maltoheptaose were used as external markers in (a) and blue dextran, glucose, maltose ... maltoheptaose as internal markers in (b).

3.8.3 Hydrolysis with Driselase

3.8.3.1 Hydrolysis of compound D

Treatment of ^{3}H-labelled compound E with Driselase will show whether compound B is part of this compound. The hydrolysis was performed over a time course (§ 2.6.3.2.1) (Fig. 3.63).

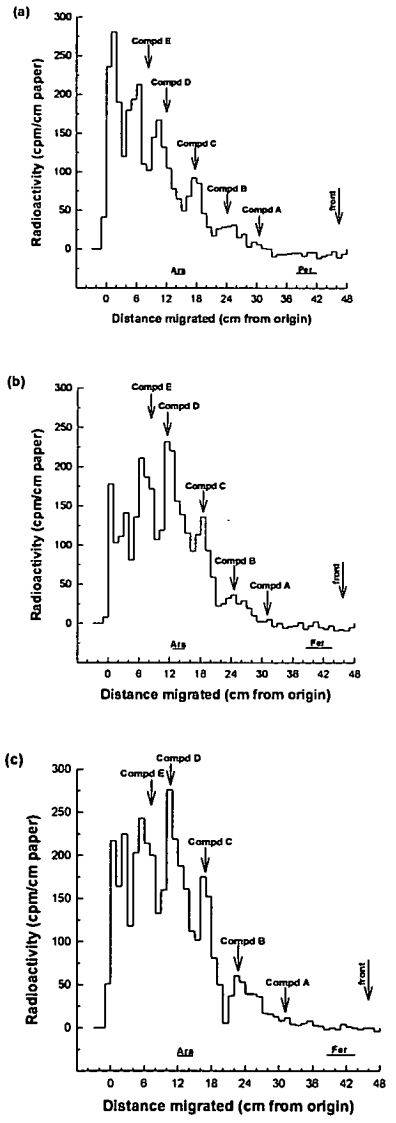


Fig. 3.63: PC in BAW (§ 2.5.1) of Driselase-hydrolysis (§ 2.6.3.2.1) products of ^{3}H-labelled compound E. (a) Hydrolysis-products after 48 h, (b) after 96 h and (c) after 144 h. Ara and Fer served as external markers. "Compd A" to "Compd E" show the approximate position of these compounds (Table 3.1, for a PC of untreated ^{3}H-labelled compound E see also figure 3.66 (a)).

3.8.3.2 Identification of compounds B and C as components of E

It is clear that ^{3}H-labelled compound E has been partially broken down by Driselase. The ^{3}H-labelled products ("compound C", 17 to 20 cm from the origin from figure 3.63 (c) and "compound B", 22 to 30 cm from the origin) were eluted (§ 2.6.4) and subjected again to PC in BAW (§ 2.5.1) with non-labelled compounds B and C as internal markers. Figure 3.64 shows that "compound C" and "compound B" co-migrated exactly with compound C and compound B as the internal markers.

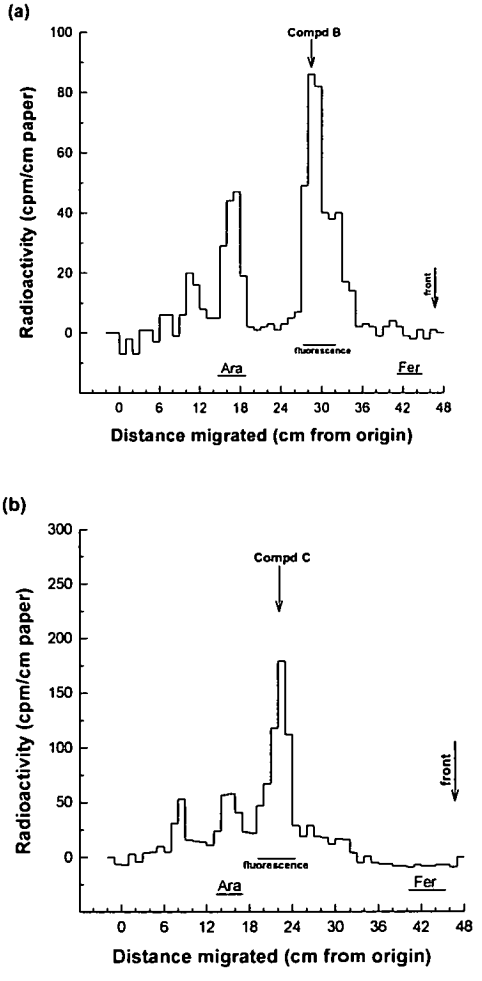


Fig. 3.64: Re-chromatography in BAW (§ 2.5.1) of (a) "compound B" (22 to 30 cm from the origin from figure 3.63 (c)) and of (b) "compound C" (17 to 20 cm from the origin). Ara and Fer were used as external and non-labelled compound B and compound C as internal markers. "Compd B" and "Compd C" show the approximate positions of these compounds (Table 3.1).

To see whether ^{3}H-labelled compound E had been broken down partially to compound D, the material (10 to 15 cm from the origin from figure 3.63 (c)) was eluted (§ 2.6.4) and purified on RPC (§ 2.6.5). The MeOH-fraction was subjected to PC in BAW (§ 2.5.1) with non-labelled compound D as internal marker (Fig. 3.65 (a)) and the H_2O-fraction to PC in EPW_1 (§ 2.5.1) (Fig. 3.65 (b). The results of figure 3.65 show that compound D was released as well as xylose and a trace of arabinose.

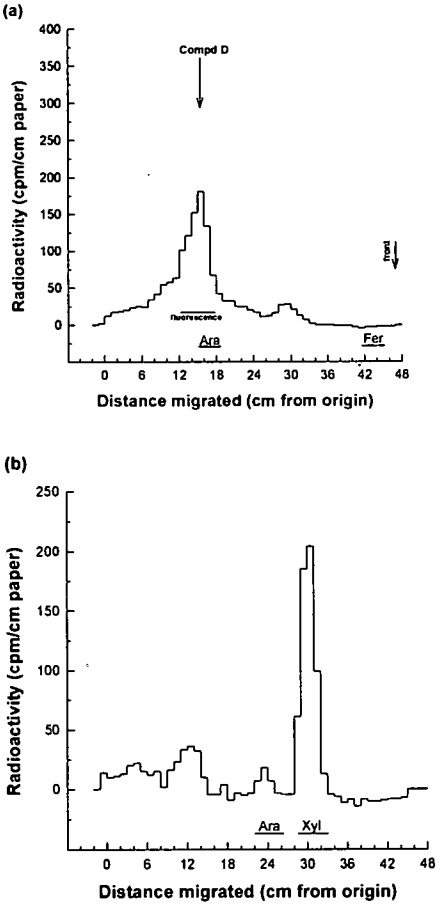


Fig. 3.65: Re-chromatography of RPC fractions of figure 3.63 (c) (10 to 15 cm from the origin). The MeOH-fraction (a) was subjected to PC in BAW (§ 2.5.1) with non-labelled compound D as internal marker and the H_2O-fraction (b) to PC in EPW_1. Ara and Xyl were used as external markers. "Compd D" shows the approximate position of this compound (Table 3.1).

The results reveal that Driselase-hydrolysis of ^{3}H-labelled compound E gave partial breakdown to compounds D, C and B, xylose and a trace of arabinose within 144 h.

3.8.4 Kinetics of alkaline hydrolysis

3.8.4.1 Determination of the half-life

To examine the alkali lability of compound E, alkaline hydrolysis was conducted with a portion of ^{3}H-labelled compound at pH ~13.3 and 25°C (§ 2.6.2) and the product(s) examined chromatographically. Figure 3.66 shows compound E after 0 min and 30 min saponification.

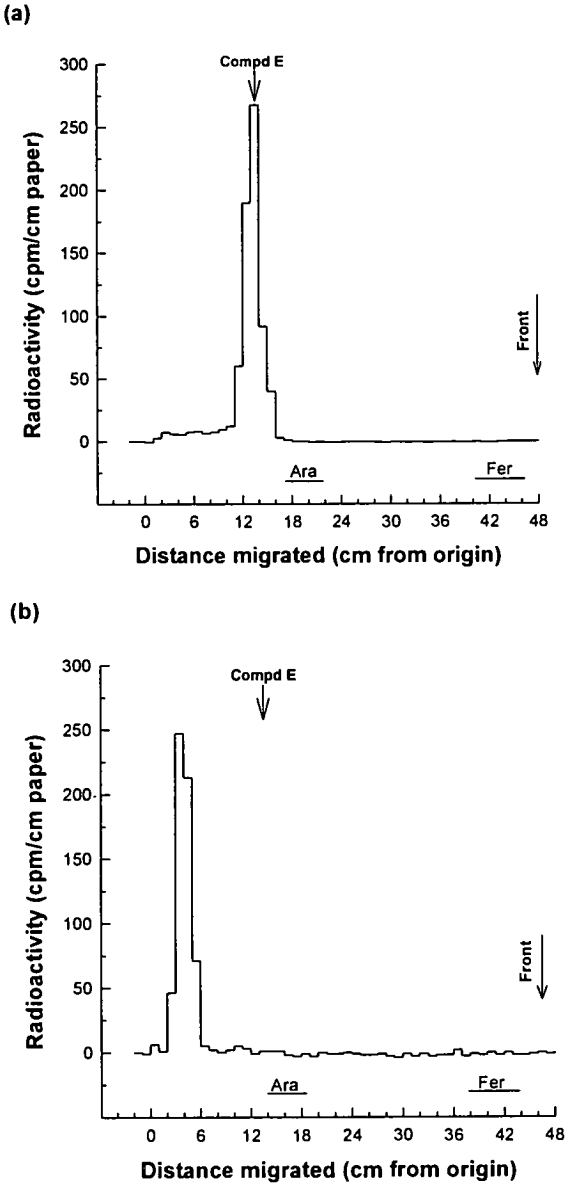


Fig. 3.66: PC in BEW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of ^{3}H-labelled compound E formed after (a) 0 min and (b) 30 min. Ara and Fer served as external markers. "Compd E" shows the approximate position of this compound (Table 3.1).

Since complete hydrolysis took place within the first 30 min (Fig. 3.66), alkaline hydrolysis of (*feruloyl*-^{14}C)-labelled compound E was carried out at pH ~13.3 and 25°C (§ 2.5.2) over a shorter time course. Figure 3.67 indicates a half-life of ~3 min and reveals that the yield of ^{14}C-ferulate after 4 min was less than the consumption of ^{14}C-labelled compound D, suggesting multiple esters.

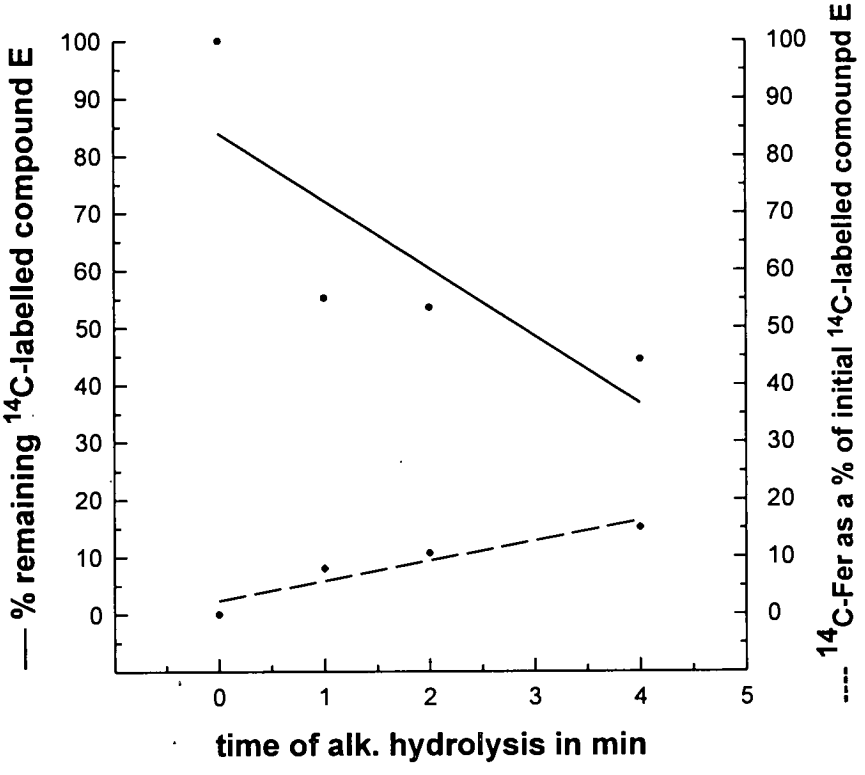


Fig. 3.67: Short-term kinetics of alkaline hydrolysis (§ 2.5.2) of ^{14}C-labelled compound E.

3.8.4.2 Test for multiple esters

To test for multiple esters in compound E, partial saponification of (*feruloyl*-^{14}C)-labelled compound E (§ 2.6.10) was carried out. Figure 3.68 shows that at least one intermediate product was formed: compound E', a product with a lower R_{Ara}-value than the original compound. It can be suggested from this that ferulate is not the sole ester-linked group in compound E.

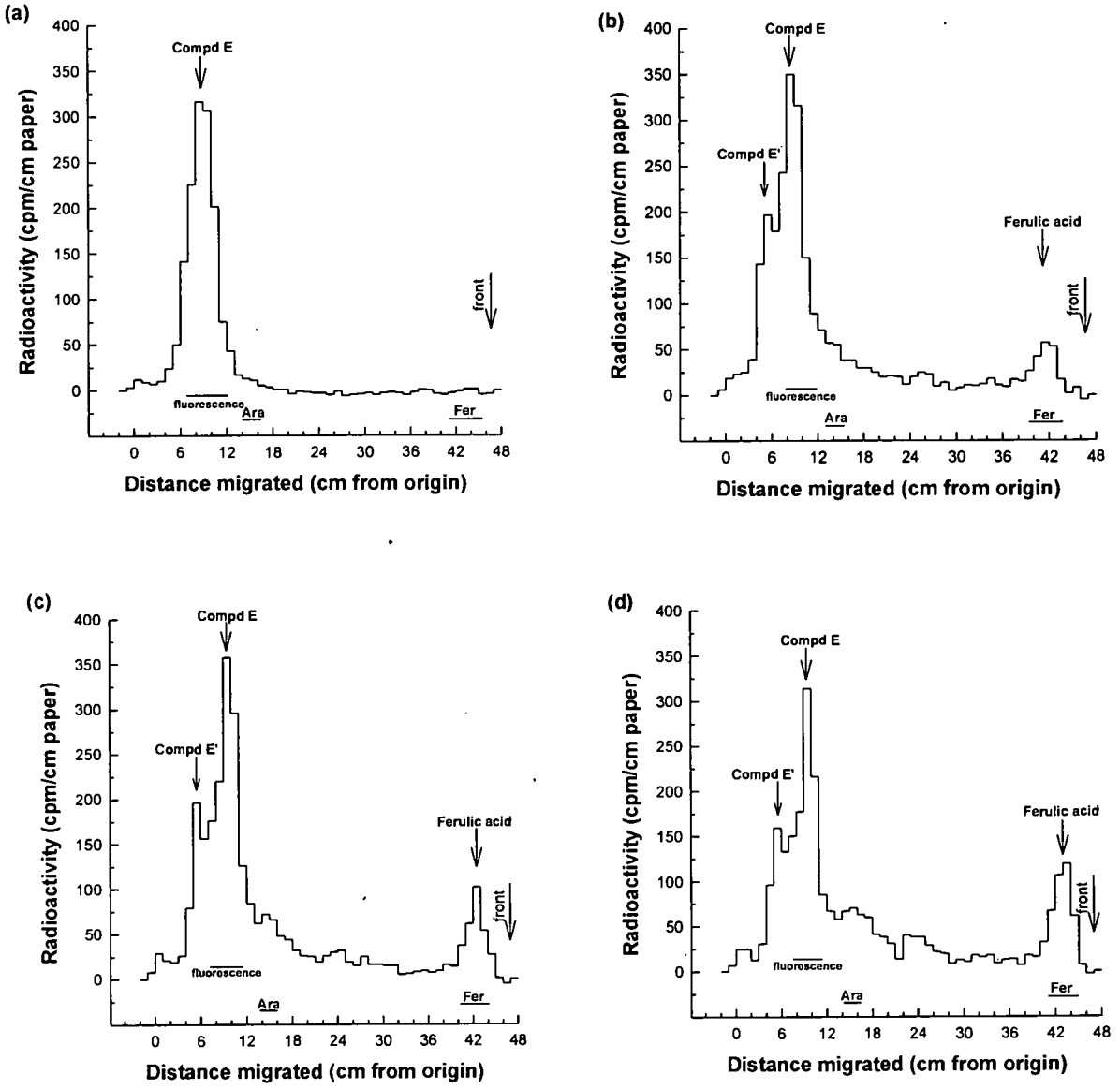


Fig. 3.68: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of ^{14}C-labelled compound E formed after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min. Ara and Fer were used as external markers. "Compd E" and "Compd E' " show the approximate position of these compounds (Table 3.1). The zero time control sample (a) received the same NaOH and HOAc as samples (b) to (d), but the HOAc was added first. All samples were enriched in phenolic-containing material by reverse-phase column chromatography (§ 2.6.5).

Complete saponification of compound E' (material from 4 to 6 cm from the origin from figure 3.68 (b)) yielded a major peak of [^{14}C]ferulate (Fig. 3.69).

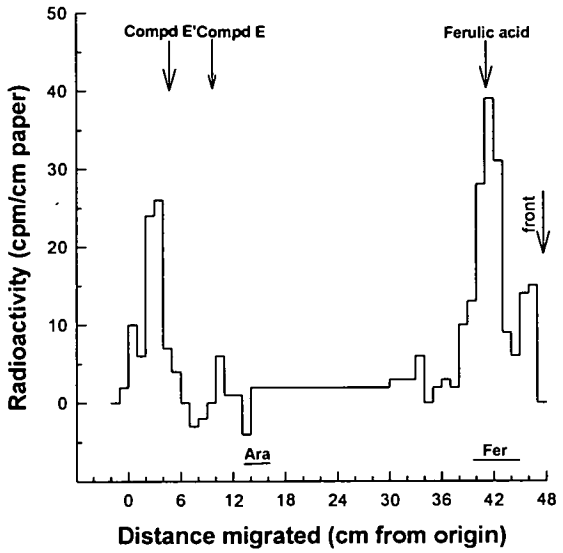


Fig. 3.69: PC in BAW (§ 2.5.1) of alkaline hydrolysis (§ 2.5.2) products of (*feruloyl*-^{14}C)-labelled compound E' from figure 3.68 (b) (material from 4 to 6 cm from the origin) formed after 1 h. Ara and Fer were used as external and Fer as internal marker. "Compd E" and "Compd E' " indicate the approximate positions of these compounds (Table 3.1).

3.8.5 Identification of the additional ester group

To test whether compound E contains an *O*-acetyl group in addition to *O*-ferulate, a suspension culture of fescue was fed with [^{14}C]acetate (see § 3.7.5). The results of figure 3.60 show that [^{14}C]acetate was successfully incorporated into compound E of fescue arabinoxylan (see also § 3.7.5).

Taken together, it is believed that compound E is a septa- or octasaccharide with a backbone of compound B. It could be shown that in addition to ferulate there is another ester-linked group present, probably acetate.

3.9 *In vivo* biosynthesis of a feruloylated arabinoxylan

3.9.1 Purification of released products after mild acid hydrolysis

To investigate *in vivo* the process of arabinoxylan feruloylation, ^{14}C-labelled ferulate and ^{3}H-labelled arabinose were fed to suspension culture cells of fescue and samples were taken over a time-course. AIR from each sample (§ 3.1) was hydrolysed with mild acid and the hydrolysate subjected to PC in BAW (§ 2.5.1). The compounds of interest were identified with the help of appropriate external markers, fluorography (of arabinose) (Figs. 3.70 and 3.71 or by their fluorescence (Fer-Ara and Fer-(Xyl)-Ara) (Fig. 3.72). The latter were also re-analysed after further purification by PC in BEW (Fig. 3.73).

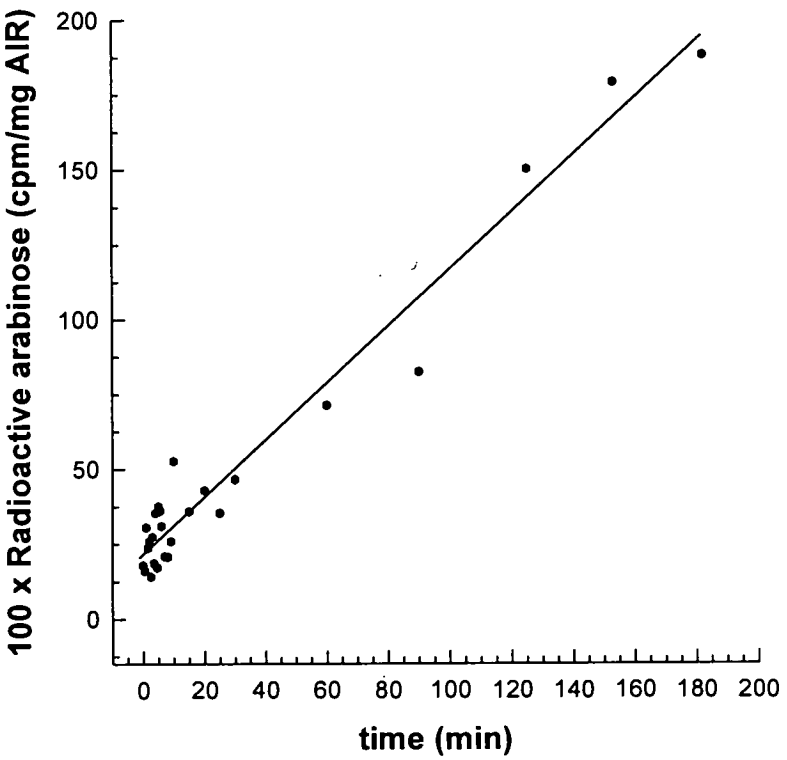


Fig. 3.70: Kinetics of incorporation of ^{3}H from [^{3}H]arabinose into AIR (Expt. 2). PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of AIR from each time point. Arabinose was eluted and assayed for radioactivity (§ 2.7.1.1).

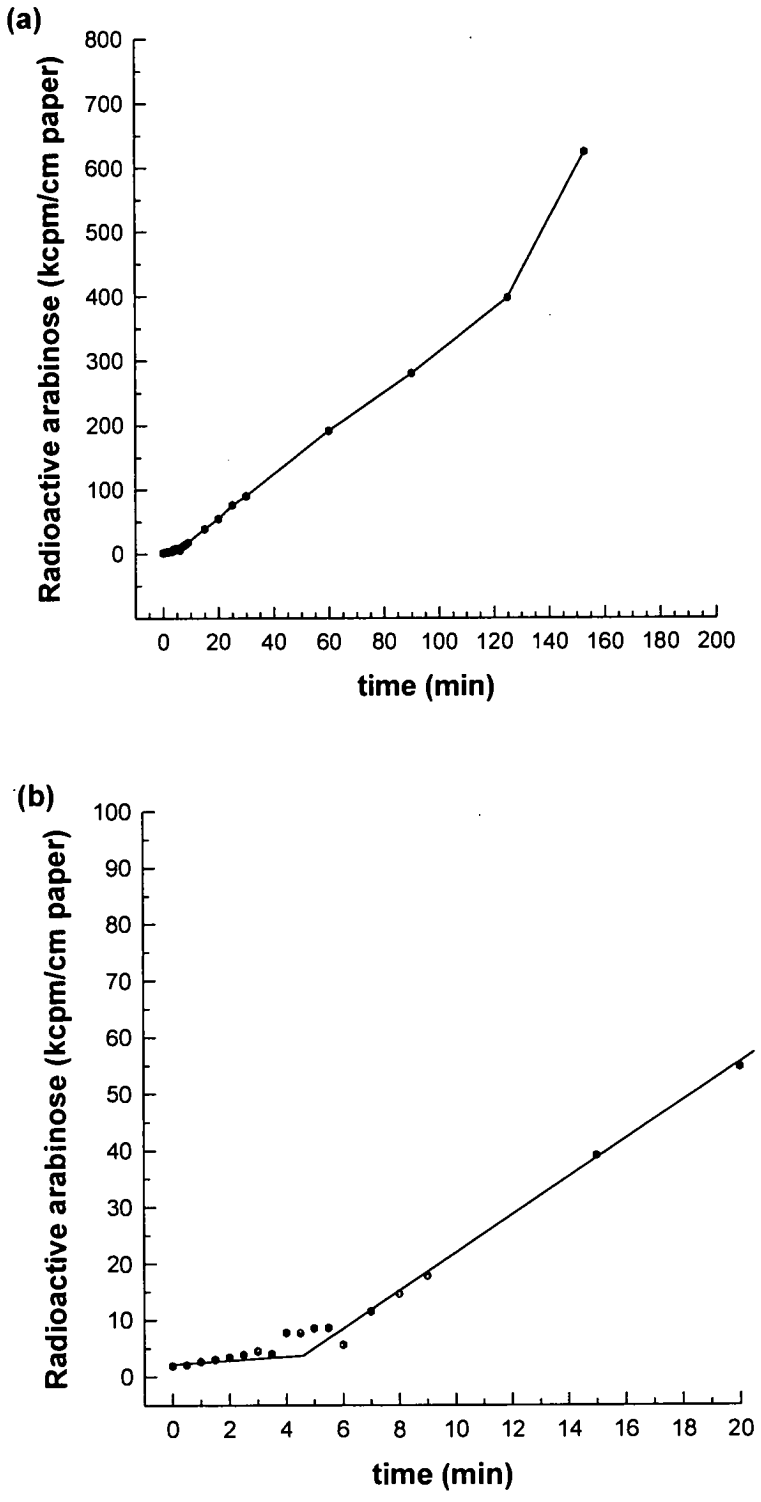


Fig. 3.71: Kinetics of incorporation of ^{3}H from [^{3}H]arabinose into AIR (Expt. 1). PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of AIR from each time point. (a) Ara was eluted and assayed for radioactivity (§ 2.7.1.1); (b) a section of (a) to clarify the initial period.

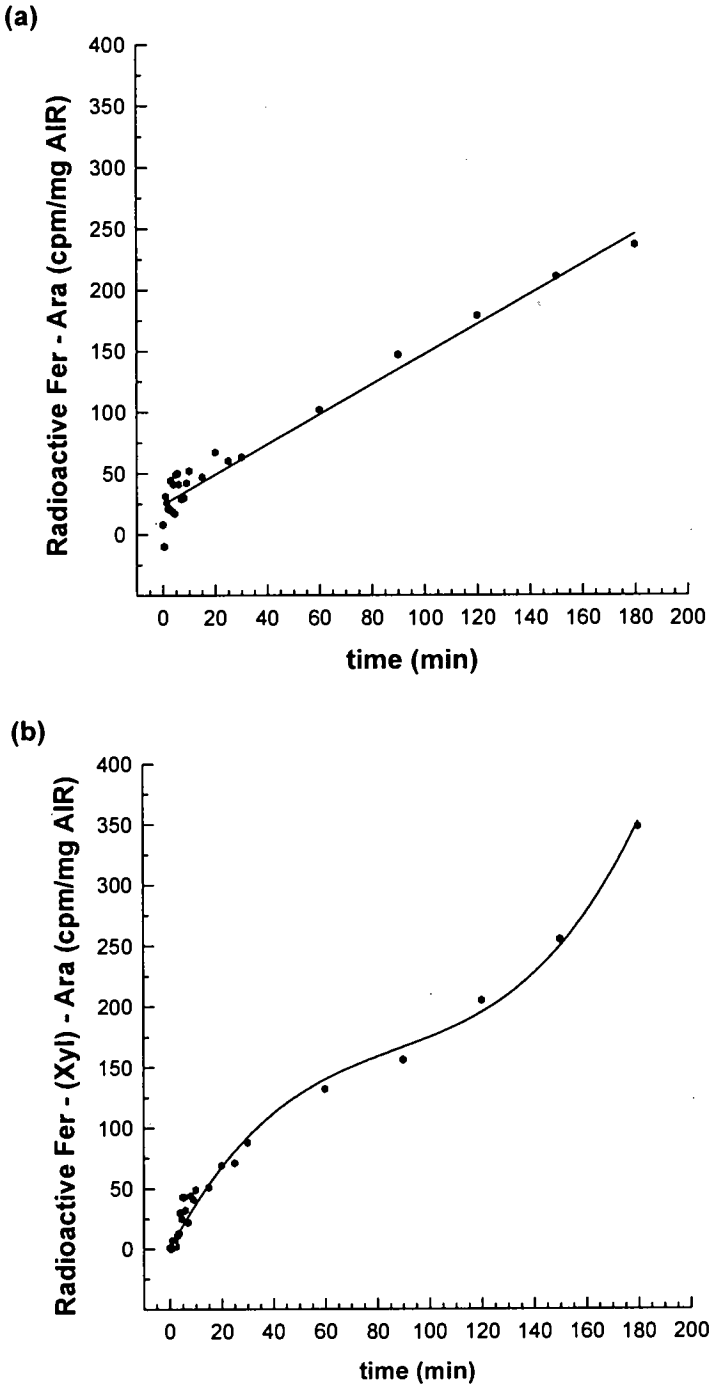


Fig. 3.72: Kinetics of incorporation of ^{14}C from [^{14}C]ferulate into ^{14}C-labelled Fer-Ara and ^{14}C-labelled Fer-(Xyl)-Ara groups of AIR. PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of AIR from each time point. Fer-Ara (a) and Fer-(Xyl)-Ara (b) were eluted and assayed for radioactivity (§ 2.7.1.1).

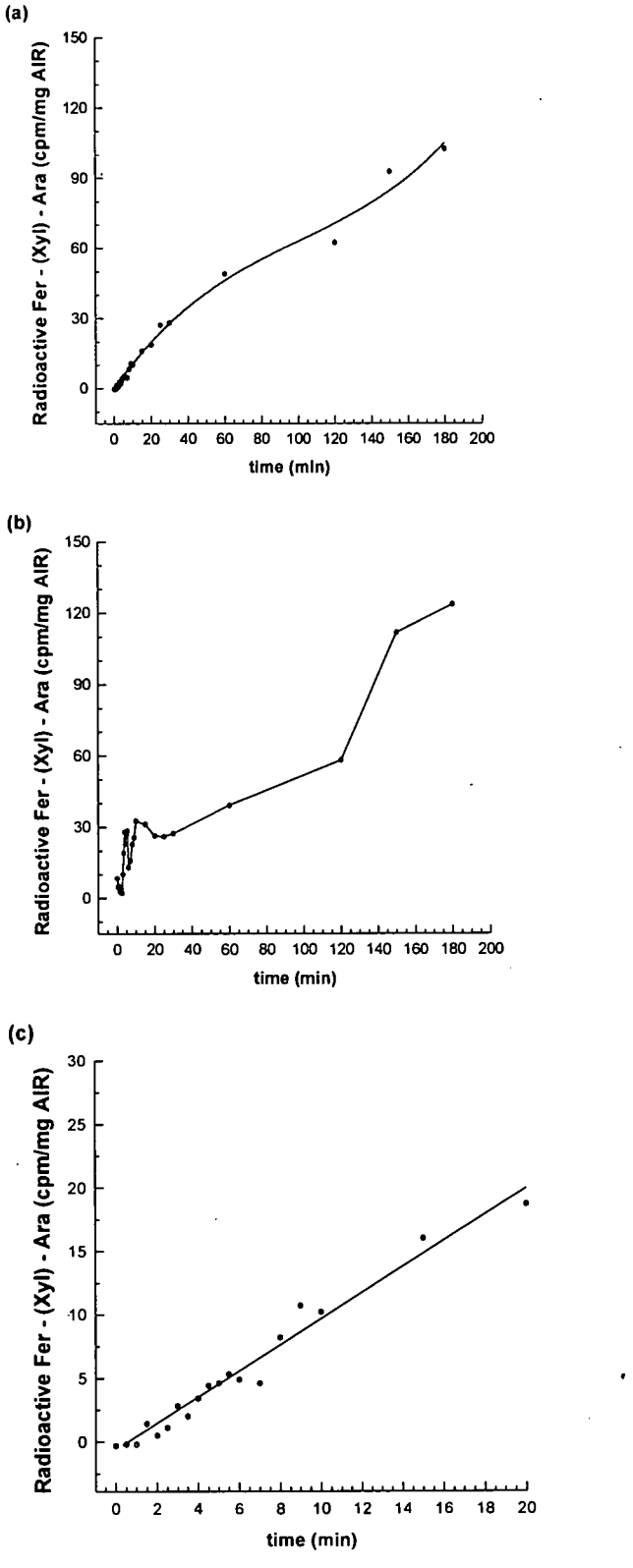


Fig. 3.73: Kinetics of incorporation of ^{14}C from [^{14}C]ferulate (a) and ^{3}H from [^{3}H]arabinose (b) into Fer-(Xyl)-Ara moieties of AIR. For these data, the Fer-(Xyl)-Ara had been further purified by PC in BEW (§ 2.5.1). (c) Shows a section of (a) to clarify the initial period.

3.9.2 Alkaline hydrolysis of Fer-Ara and Fer-(Xyl)-Ara

Alkaline hydrolysis (§ 2.6.2) of Fer-Ara and Fer-(Xyl)-Ara was performed and the released products were assayed for radioactivity (§ 2.7.1.1) (Figs. 3.74, 3.75 and 3.76).

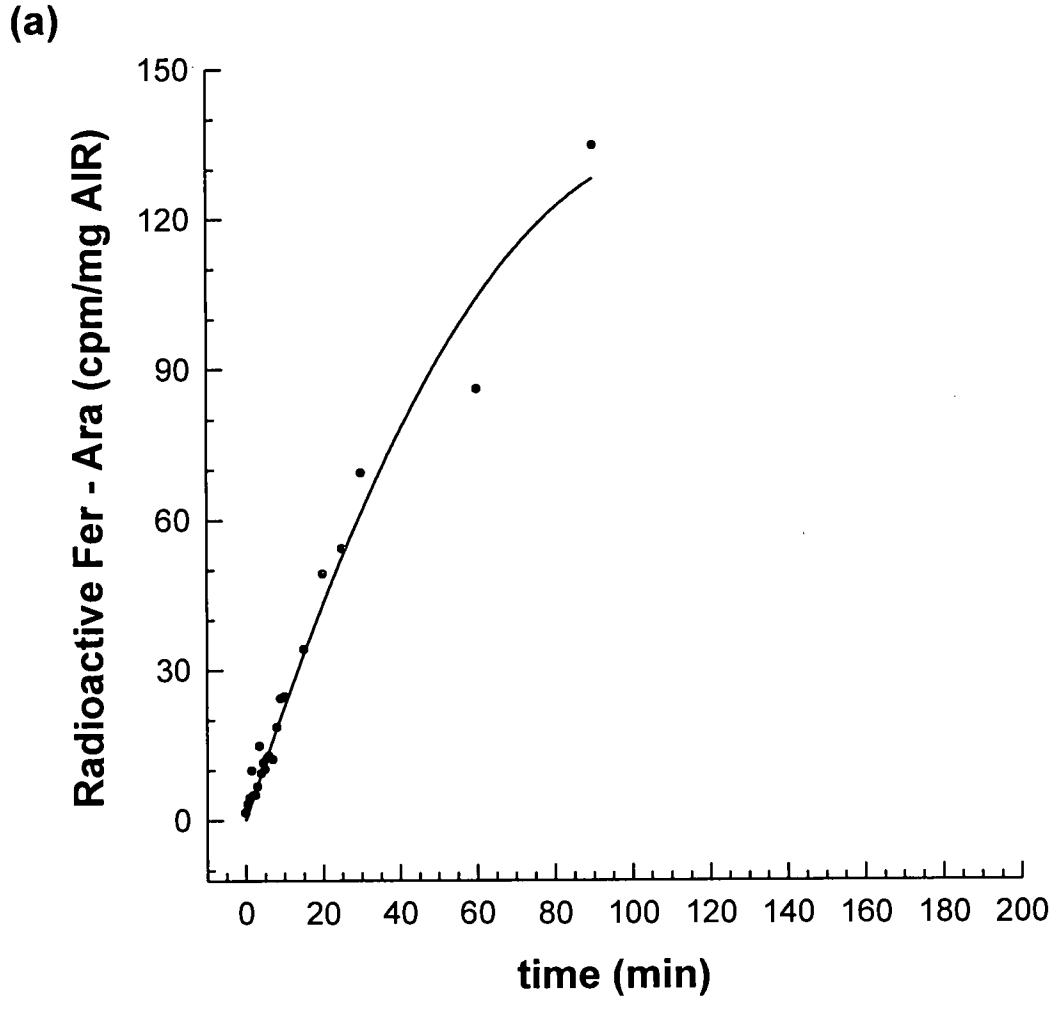


Fig. 3.74: Kinetics of labelling of alkaline hydrolysis (§ 2.6.2) products of Fer-Ara. Hydrolysis products were subjected to PC in BAW (§ 2.5.1) and assayed for ^{14}C.

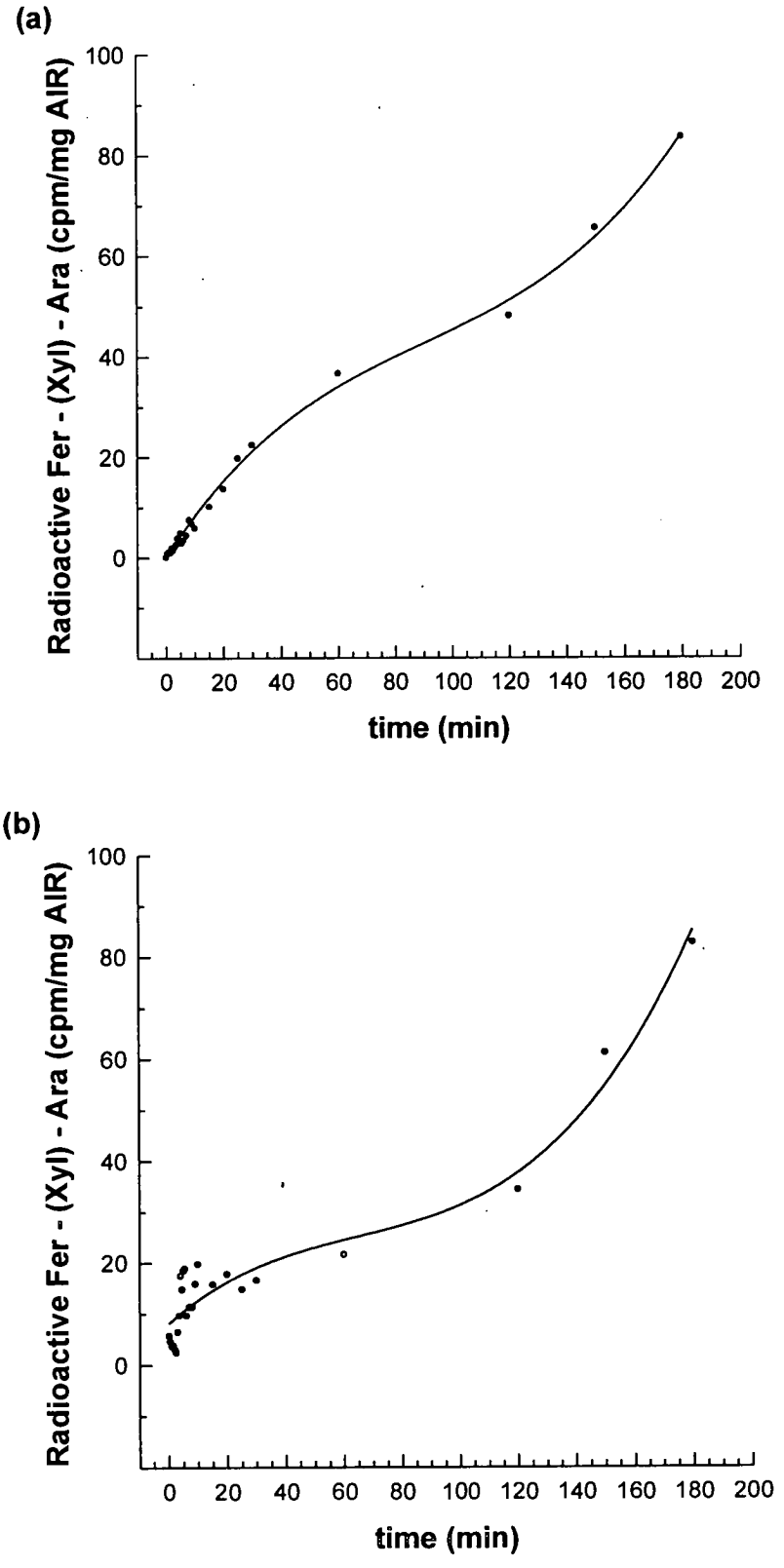


Fig. 3.75: Kinetics of labelling of alkaline hydrolysis (§ 2.6.2) products of Fer-(Xyl)-Ara. Hydrolysis products were subjected to PC in BAW (§ 2.5.1) and assayed for (a) ^{14}C and (b) ^{3}H.

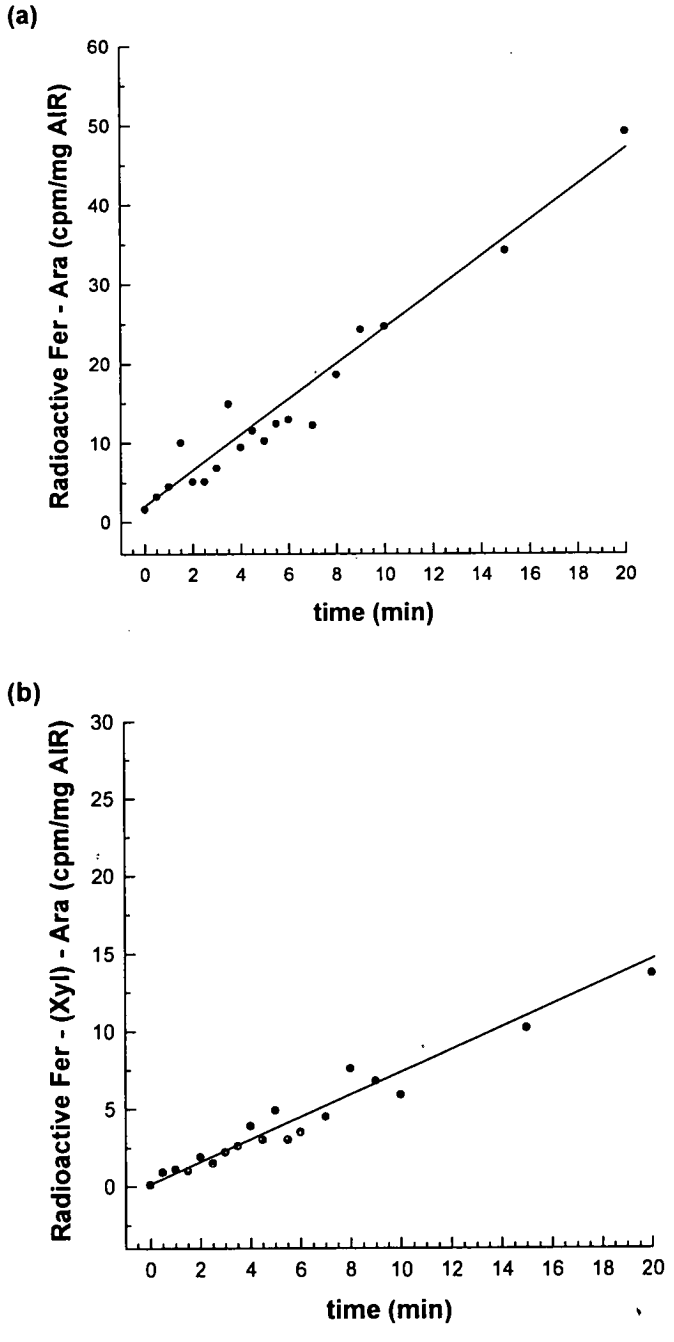


Fig. 3.76: Short-term kinetics of labelling of the feruloyl moiety of Fer-Ara (a) and Fer-(Xyl)-Ara (b). (a) Shows a section of figure 3.74, and (b) shows a section of figure 3.75 (a); to clarify the initial period.

In the *in vivo* biosynthesis of a feruloylated arabinoxylan of fecsue it could be demonstrated that both the Fer-Ara and Fer-(Xyl)-Ara groups became ^{3}H-labelled with a lag of <5 min and ^{14}C-labelled with a lag of <1 min indicating that the arabinose is first xylosylated and then feruloylated.

3.10 Investigation of *O*-feruloylated oligosaccharides released from fescue and maize cell walls by Driselase

3.10.1 Treatment of non-labelled AIR with Driselase

3.10.1.1 Calculation of the percentage of soluble products from fescue AIR

Non-radioactive AIR was prepared from freeze-dried cultured cells of fescue (§ 2.5.10.2). A portion was hydrolysed with Driselase and the soluble products were saponified (§ 2.6.9.1). The A_{343} was taken as a measure of the ferulate content (ε=21000 $M^{-1}cm^{-1}$). The Driselase-insoluble residue was also assayed by A_{343}. Two preparations served as controls: cell walls incubated alone in buffer and then treated with alkali, and Driselase incubated alone in buffer and then treated with alkali. Table 3.14 indicates that the recovery of ferulate in the soluble and insoluble fractions of the Driselase digest was: (79.7 ÷ 73.9) x 100 = 108%. The proportion of ferulate groups that had been released as soluble conjugates by Driselase was: [(103 – 39.6) ÷ 79.7] x 100 = 79.5%.

Treatment	A_{343}*	Concentration of Na-ferulate in alkaline hydrolysate (µM)**	Yield of Na ferulate (nmol) per 10 mg AIR and/or 2.5 mg Driselase
AIR + Driselase			
(a) soluble products	1.98	94.1	103
(b) insoluble products	0.68	32.6	16.3
(c) Driselase alone	0.83	39.6	39.6
(d) = [(a) + (b)] - (c)			79.7
(e) AIR alone	3.10	147.8	73.9

Table 3.14: Calculation of the feruloylated material that could be solubilised from 10 mg fescue AIR. AIR was hydrolysed by Driselase: soluble and insoluble products were subjected to alkaline hydrolysis and the A_{343} was measured. AIR alone and Driselase alone served as controls.

* 4 x A_{343} of a 4-fold dilution;

** as sodium ferulate: ε=21000 $M^{-1}cm^{-1}$

3.10.1.2 Calculation of the percentage of soluble products from maize AIR

Non-radioactive AIR of maize was prepared as described in § 2.6.9.1 and 3.10.1.1. A portion was hydrolysed with Driselase followed by treatment as described (§ 3.10.1.1). Table 3.15 indicates that the recovery of ferulate in the soluble and insoluble fractions of the Driselase digest was: (410.8 ÷ 353.4) x 100 = 116%. The proportion of ferulate groups that had been released as soluble conjugates by Driselase from maize AIR was: [(362 – 39.6) ÷ 410.8] x 100 = 78.5%.

Treatment	A_{343}	Concentration of Na-ferulate in alkaline hydrolysate (µM)***	Yield of Na-ferulate (nmol) per 10 mg AIR and/or 2.5 mg Driselase
AIR + Driselase			
(a) soluble products	6.34*	301.6	362
(b) insoluble products	3.71*	176.7	88.4
(c) Driselase alone	0.83*	39.6	39.6
(d) = [(a) + (b)] - (c)			410.8
(e) AIR alone	14.85**	706.8	353.4

Table 3.15: Calculation of the feruloylated material that could be solubilised from 10 mg maize AIR. Details as in Table 3.14.
* 8 x A_{343} of an 8-fold dilution,
** 64 x A_{343} of a 64-fold dilution;
***as sodium ferulate: ε=21000 $M^{-1}cm^{-1}$.

3.10.1.3 Pattern of soluble products released from fescue AIR by Driselase

In order to see how much of the products were oligomeric rather than polymeric, a portion of the soluble products released by Driselase from fescue AIR (2 ml ≡ 36.4 mg AIR and 9.1 mg Driselase) (§ 3.10.1.1) was

mixed with 500 µl formic acid and subjected to PC (as a 37-cm streak) in BAW (§ 2.5.1). Strips of the paper were treated with NaOH and the absorbance was measured at 343 nm (Fig. 3.77). The absorbance of a Driselase-alone control was subtracted (from each strip) for the calculation of the percentage of oligomeric material.

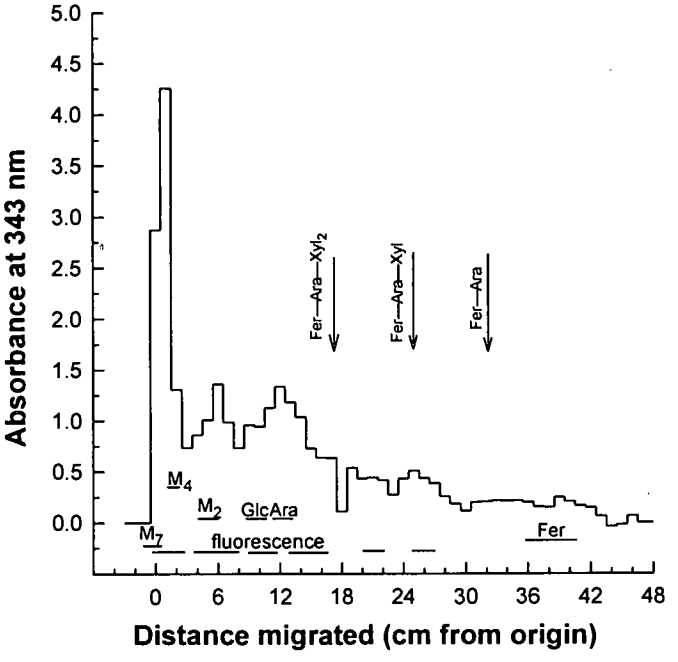


Fig. 3.77: PC in BAW (§ 2.5.1) of soluble Driselase-digestion products of fescue AIR. 1-cm Strips were treated with 0.5 M NaOH for 1 h and the absorbance was measured at 343 nm. Fer, Ara, Glc, M_2, M_4, and M_7 served as external markers. "Fer-Ara (compd A)", "Fer-Ara-Xyl (compd B)" and "Fer-Ara-Xyl_2" indicate their approximate position (Table 3.1).

About 72% of the Driselase-solubilised material that, on saponification, gave material absorbing at 343 nm, was judged to be oligomeric (i.e. $R_{Ara} > 0.16$) rather than polymeric. Most of the oligomeric material was of relatively high molecular weight as judged by its position relative to compds A (Fer-Ara) and B (Fer-Ara-Xyl). A feruloylated trisaccharide (Fer-Ara-Xyl_2) has an R_{Ara}-value of 1.18 (Fry, 1988). The fact that most of the oligomeric material was of relatively high molecular weight has to be seen in contrast to maize cell walls where most of the material was released as FAXX .

3.10.1.4 Pattern of soluble products released from maize AIR by Driselase

In order to calculate the percentage of the soluble products which were oligomeric rather than polymeric, 2.184 ml (≡ 36.4 mg AIR and 9.1 mg Driselase) of Driselase-hydrolysed maize AIR was treated as described in § 3.10.1.3 (including the control). Figure 3.78 shows the pattern of soluble products.

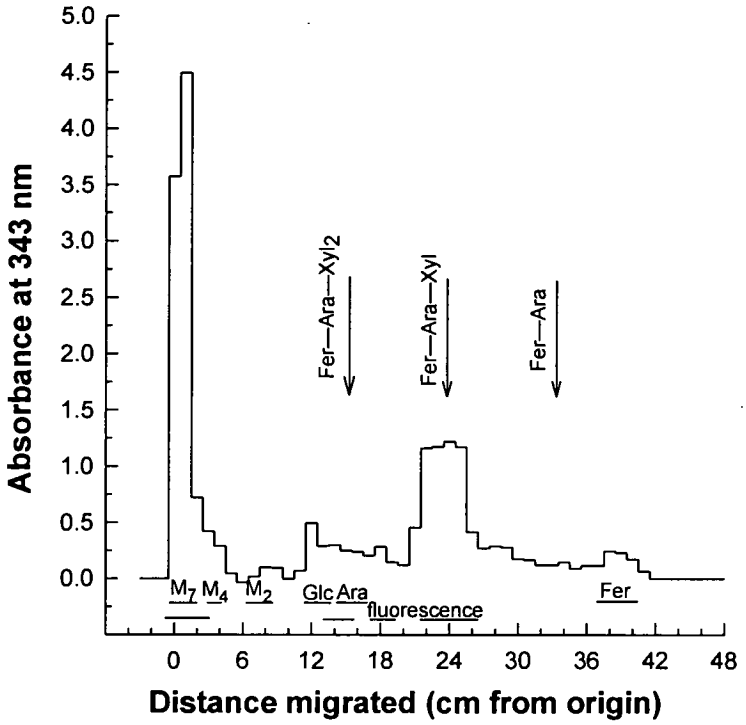


Fig. 3.78: PC in BAW (§ 2.5.1) of soluble Driselase-digestion products of maize AIR. 1-cm Strips were treated with 0.5 M NaOH for 1 h and the absorbance was measured at 343 nm. Fer, Ara, Glc, M_2, M_4, and M_7 served as external markers. Fer-Ara (compd A), Fer-Ara-Xyl (compd B) and Fer-Ara-Xyl_2 indicate their approximate position.

About 55.8% of the Driselase-solubilised material that, on saponification, gave material absorbing at 343 nm, was judged to be oligomeric (i.e. $R_{Ara} > 0.16$) rather than polymeric; most of the oligomeric material was of relatively low molecular weight, probably FAXX.

3.10.2 Treatment of ^{3}H-labelled fescue AIR by Driselase

3.10.2.1 Preparation of ^{3}H-labelled Driselase released products

A portion of ^{3}H-labelled fescue AIR (§ 3.1) was incubated with Driselase and the hydrolysate subjected to PC in BAW (§ 2.5.1) (Fig. 3.79).

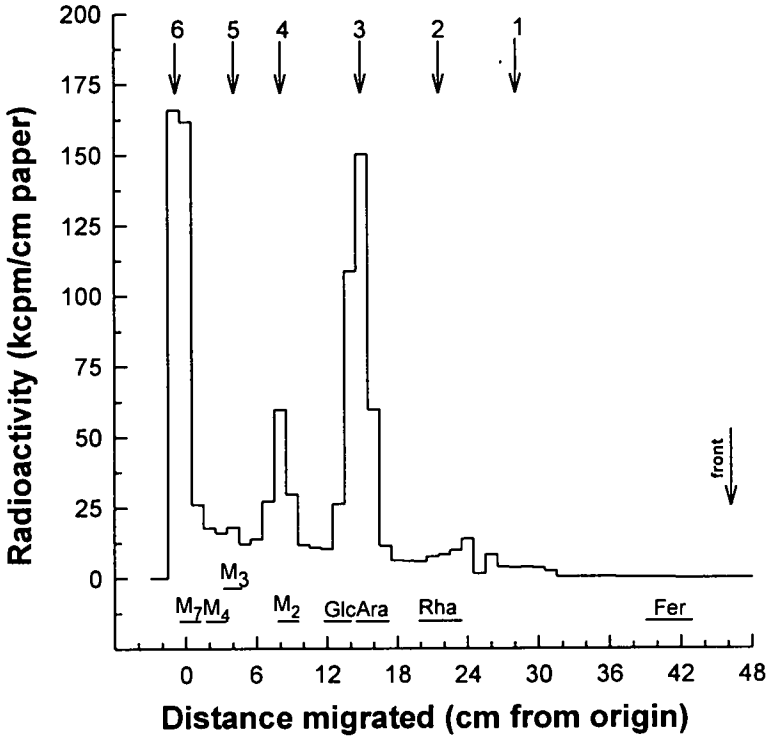


Fig. 3.79: PC in BAW (§ 2.5.1) of Driselase-digestion products of ^{3}H-labelled fescue AIR. Six blue fluorescent zones (1 to 6) were located. The arrows indicate the middle of each zone. M_7, M_4, M_3, M_2, Glc, Ara, Rha and Fer served as external markers.

Six blue-fluorescing zones (compounds 1 to 6) were located by brief exposure to a 366-nm UV-lamp. The fluorescence turned an intense blue-green under ammonia vapour indicating the presence of feruloyl esters (see also § 3.1). To remove the large amount of ^{3}H-labelled mono- and disaccharides (Fig. 3.79), the aromatic-containing material present in the digest was purified by RPC as described in § 3.1.1 (Fig. 3.80). Table 3.16 summarises the chromatographic data of the 6 fluorescent compounds.

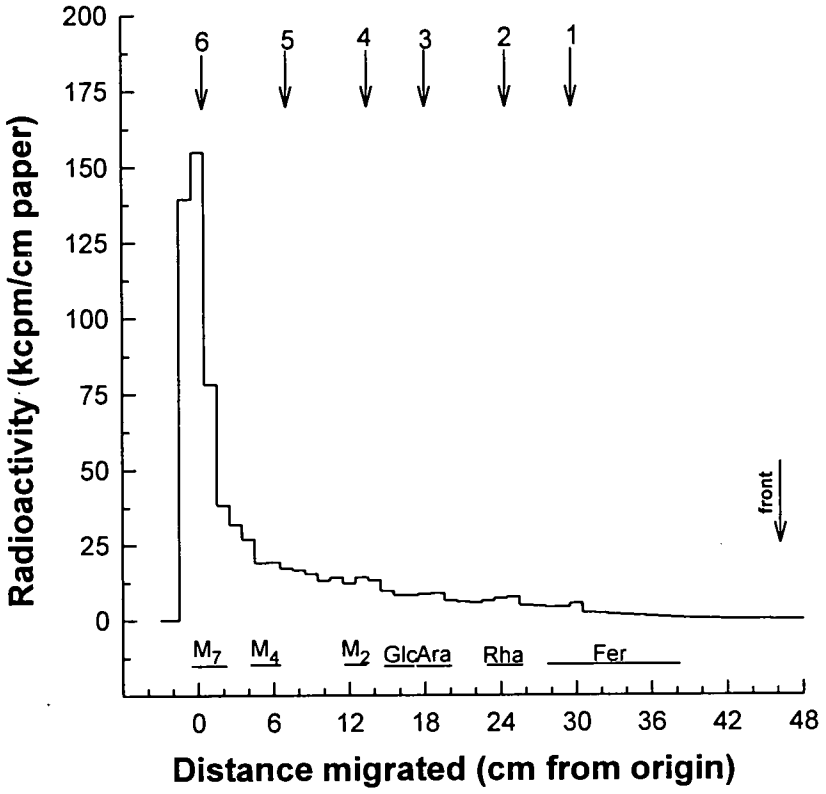


Fig. 3.80: PC in BEW (§ 2.5.1) of Driselase-digestion products of ^{3}H-labelled fescue AIR after further purification by RPC (§ 3.1.1). Six blue fluorescent zones (1 to 6) were located. The arrows indicate the middle of each zone. M_7, M_4, M_2, Glc, Ara, Rha and Fer served as external markers.

Compound	R_{Ara} (in BAW)	R_{Ara} (in BEW)
1	1.88	1.63
2	1.38	1.37
3	0.88	1.05
4	0.50	0.74
5	0.31	0.37
6	0.00 - 0.05	0.00 - 0.05

Table 3.16: Chromatographic data (R_{Ara}-values) obtained for compds 1 to 6 by PC in BAW and BEW (§ 2.5.1).

3.10.2.2 Proposed structures of compounds 1, 2 and 3

The proposed structures of compounds 1, 2 and 3 are given in figure 3.81.

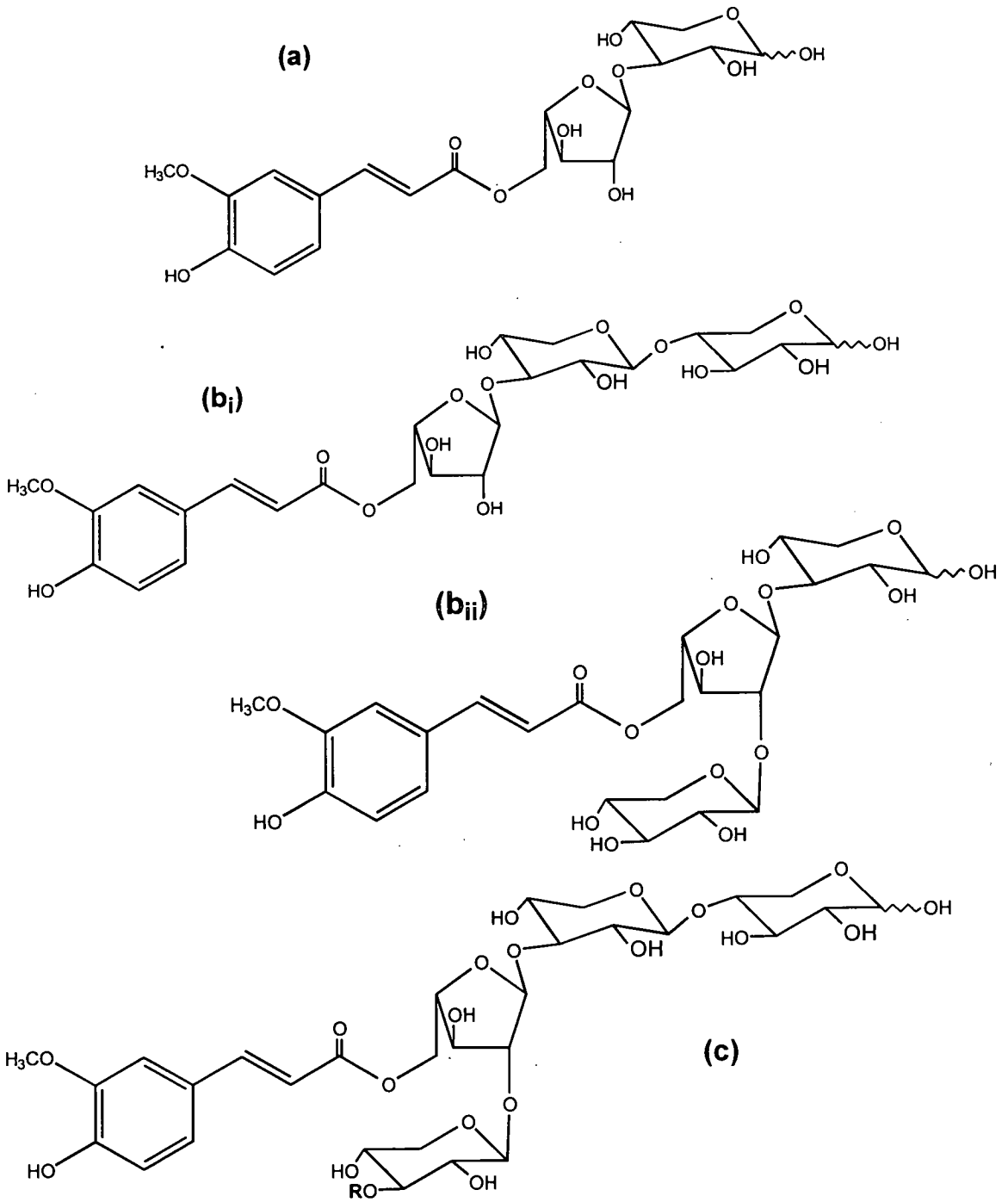


Fig. 3.81: The proposed structures of compounds 1 (a), 2 (b) and 3 (c).

3.10.2.3 Complete acid hydrolysis

Portions of de-feruloylated ^{3}H-labelled compounds 1, 2 and 3 (i.e. compds 1_S, 2_S and 3_S) were subjected to complete acid hydrolysis (§ 2.6.1) and the products were separated by PC in EPW_1 (§ 2.5.1), yielding arabinose and xylose (Fig. 3.82, Table 3.17).

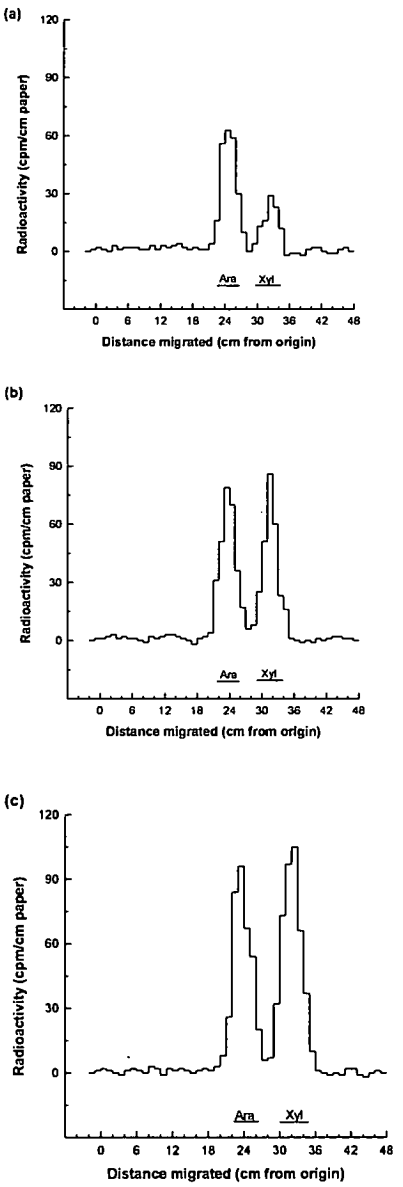


Fig. 3.82: PC in EPW_1 (§ 2.5.1) of complete acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compounds 1_S (a), 2_S (b) and 3_S (c). Ara and Xyl served as external and internal markers.

In addition, a portion of de-feruloylated ^{3}H-labelled compound 2 (compound 2_S) was reduced (§ 2.6.6) before treatment with 2 M TFA (§ 2.6.1) and analysed as above (Fig. 3.83, Table 3.17).

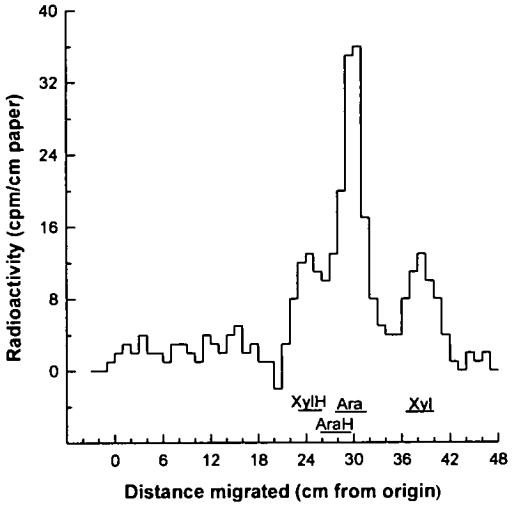


Fig. 3.83: PC in EPW_1 (§ 2.5.1) of complete acid hydrolysis (§ 2.5.1) products of ^{3}H-labelled, reduced (§ 2.6.6) compound 2_S. Ara, AraH, Xyl and XylH served as external markers.

On the basis of molar Ara : Xyl ratios, it can be suggested that compound 1_S is a disaccharide (Ara_1, Xyl_1), compound 2_S a trisaccharide (Ara_1, Xyl_2) and compound 3_S a tetrasaccharide (Ara_1, Xyl_3). Borohydride reduction of 2_S yielded [^{3}H]xylitol (Fig. 3.83), indicating that xylose was its reducing terminus.

3.10.2.4 Mild acid hydrolysis

Mild acid hydrolysis (§ 2.6.1) of Driselase-released products is expected to set free the part of each compound that originated from the xylan backbone (xylose, xylobiose, xylotriose ...), leaving a feruloylated oligomer with arabinose as its reducing terminus.

Predicted products of ^{3}H-labelled compound 1 after treatment with 0.1 M TFA would be ^{3}H-labelled compound A and [^{3}H]xylose. Indeed, figure 3.84 shows radiolabelled products compatible with this prediction (free Ara does

not resolve from Xyl on PC in BAW). In addition, a disaccharide was set free; probably Ara-Xyl. Cleavage of the ester-bond in compound 1 would result in a disaccharide (Ara-Xyl) (see also § 3.10.2.5). However, 0.1 M TFA would quickly hydrolyse such a disacccharide. It can thus be suggested that compound 1 was contaminated with small amounts of compound 2 [Xyl - (Fer-Ara)-Xyl], which on mild acid hydrolysis would yield some Xyl-Ara.

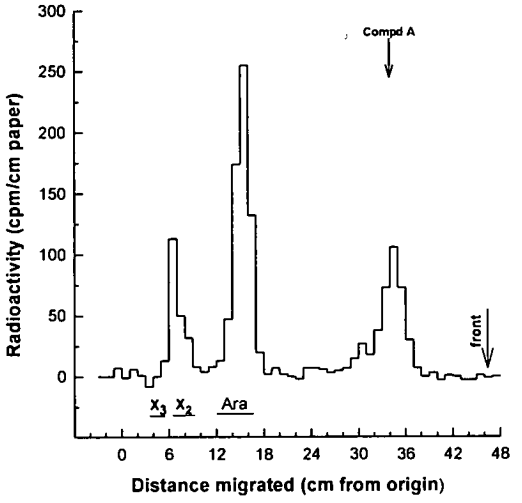


Fig. 3.84: PC in BAW § 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compound 1. Ara, X_2 and X_3 served as external markers. "Compd A" shows the approximate position of this compound (Table 3.1).

Treatment of compound 2 with mild acid resulted in the release of equal amounts (on a ^{3}H basis) of compounds that co-chromatographed with compounds A and B, respectively (Fig. 3.85 (a)) as well as putative mono-, di- and trisaccharides and a trace of remaining compound C (material from strips 21 to 23 cm from the origin). Material from strips 13 to 19 cm from the origin is believed to include both xylose and arabinose. From a compound with the structure given in figure 3.81 (b_{ii}), equal yield of compound A and xylobiose would have been expected. However, no explanation could be found for the material at the origin. The appearance of equal amounts of compounds A and B is only compatible with a mixture of compounds with the structures shown in figure 3.81 (b).

Ishii & Saka (1992) reported from a feruloylated trisaccharide with the structure shown in figure 3.81 (b_i) to inhibit auxin-stimulated elongation.

Treatment of compound 3 under the same conditions as described above appeared to give compound C as the major feruloylated oligosaccharide (Fig. 3.85 (b)). The smaller amounts of compounds A and B would arise by partial hydrolysis of the feruloyl trisaccharide. Together with the appearance of a disaccharide, tentatively identified as xylobiose as the major de-feruloylated oligosaccharide (material at 10 to 12 cm from the origin in figure 3.85 (b)), the structure given in figure 3.81 (c) is postulated.

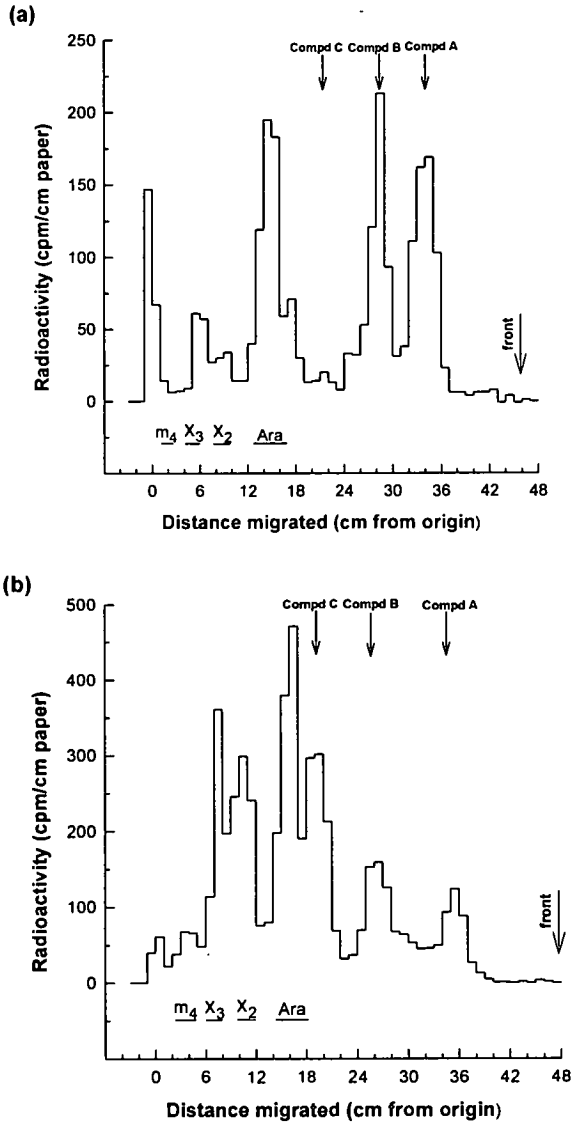


Fig. 3.85: PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of ^{3}H-labelled compounds 2 (a) and 3 (b). Ara, X_2, X_3 and m_4 served as external markers. "Compd A", "Compd B" and "Compd C" show the approximate position of these compounds (Table 3.1).

3.10.2.5 Mild acid hydrolysis of compound B

Since compounds A to H (§ 3.1) can be released by mild acid hydrolysis, it is very likely that the reducing terminal sugar of each compound had been linked in its furanose form within its parent polymer (see also introduction). To test to what extent other linkages will be cleaved as well by the mildly acidic conditions used, ^{3}H-labelled compound B was treated with 0.1 M TFA for 1 h and the released products were separated by PC in BAW (§ 2.5.1). Together with data obtained from figure 3.86, the percentage was estimated as 100% Ara_f, 60% Xyl_p and 10% Fer.

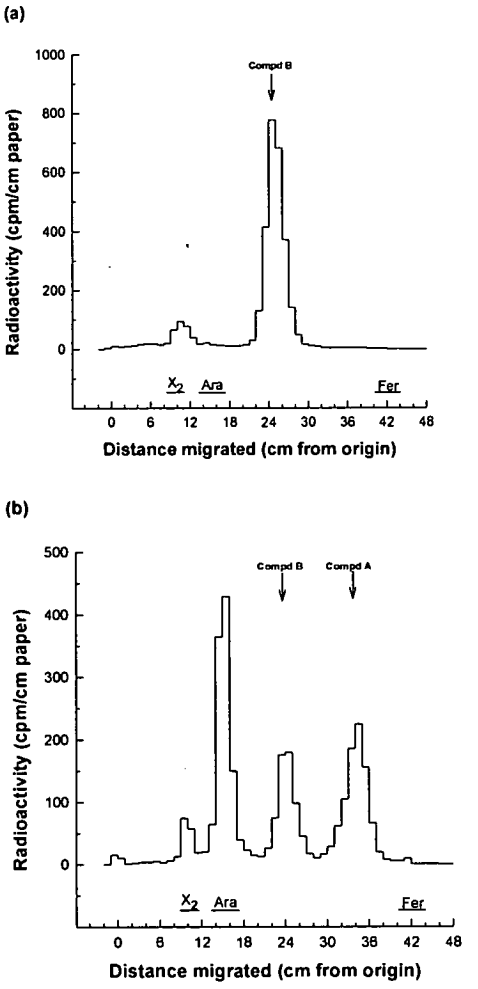


Fig. 3.86: PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of (*pentosyl*-^{3}H-labelled compound B. (a) Compound B without any treatment and (b) compound B after treatment with 0.1 M TFA for 1 h. Ara, X_2 and Fer served as external markers. "Compd A" and "Compd B" show the approximate position of these compounds (Table 3.1).

3.10.2.6 Attachment of feruloyl-arabinose to the xylan backbone

The compounds that were released by mild acid hydrolysis (§ 3.1) could have been connected to either *O*-2 or *O*-3 of a xylose residue of the xylan backbone. In figure 3.13 (§ 3.5.1) a (1→3)-linkage is assumed. It is expected that Driselase will cleave the xylan backbone without splitting the bond between a feruloylated Ara residue and the Xyl of the backbone (see also previous section). Furthermore, compound 3 appears to have a xylobiose group from the xylan backbone.

To distinguish between (1→2)- and (1→3)-linkages, Smith degradation (§ 2.6.7) was carried out with a portion of compound 3. If the arabinose residue is linked to *O*-2' of a [^{3}H]xylobiose group (of the backbone), $NaIO_4$-oxidation followed by TFA-hydrolysis will yield [^{3}H]formic acid and [^{3}H]glyceraldehyde (from the backbone) and [^{3}H]arabinose (see discussion, § 4.5). In the case of a linkage to *O*-3' of the [^{3}H]xylobiose moiety, we would expect to obtain [^{3}H]formic acid and [^{3}H]xylose (from the backbone) and [^{3}H]arabinose. Figure 3.87 shows arabinose and xylose as major radioactive products. The significance of the Ara : Xyl ratio is discussed in § 4.5.

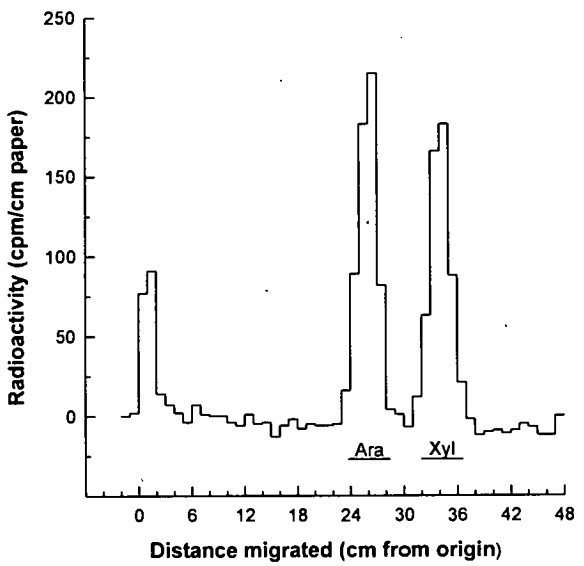


Fig. 3.87: PC in EPW_1 (§ 2.5.1) of hydrolysate of ^{3}H-labelled compound 3 after Smith degradation (§ 2.6.7). Ara and Xyl served as external and internal markers.

From the results of the Smith degradation we can deduce a (1→3)-linkage of the feruloylated arabinose residue to the xylan backbone (see discussion). Table 3.17 summarises the Ara : Xyl ratios of compounds 1_S, 2_S and 3_S and of compound 3 after Smith degradation.

compound	Ara : Xyl ratio				Ara : Xyl/XylH ratio	
	after 2 M TFA		after smith degradation		after $NaBH_4$-red. and 2 M TFA	
	on ^3H-basis	on molar basis *	on ^3H-basis	on molar basis *	on ^3H-basis	on molar basis *
1_S	2.412	0.955	nd	nd	nd	nd
2_S	1.088	0.431	nd	nd	1.299	0.515
3_S	0.826	0.327	nd	nd	nd	nd
3	nd	nd	1.107	0.439	nd	nd

Table 3.17: Calculation of the Ara : Xyl and Ara : Xyl/XylH ratios after complete acid hydrolysis of compounds 1_S, 2_S and 3_S and of $NaIO_4$-oxidised compound 3.

* Molar ratios were calculated taking into account the specific radioactivity values given in § 3.6.2.

The molar arabinose : xylose ratio of compound 3_S is 1 : 3, suggesting a feruloylated tetrasaccharide and after Smith degradation of compound 3 the ratio was approximately 1 : 2. The latter ratio is only possible if compound 3 has two periodate-resistant Xyl residues. One of these would be the non-reducing Xyl residue of the xylobiose group. The second would be the Xyl residue of the compound B moiety. The "R" group (Fig 3.81 (c)) at position *O*-3 of the latter Xyl residue could be another sugar so that compound 3 is a pentasaccharide. It was found that the R_{Ara}-values (in BAW) of compound 3 and compound D are very similar (0.88 and 0.95, respectively). Compound D was suggested to be a feruloylated pentasaccharide (Fig. 3.51). "R" can also be an *O*-acetyl group. However, cleavage of the furanosyl linkage in compound 3 would then result in a disaccharide carrying both a feruloyl and an acetyl ester. Such compounds have not been discovered in fescue arabinoxylan; only compounds with >3 sugar units were found to be dual esters (see §§ 3.7.5 and 3.8.5).

3.11 Treatment of plant cell wall (PCW) fragments with rat caecal bacteria

3.11.1 Treatment of [^{3}H]arabinose with rat caecal bacteria

In view of the unusual structure of compound B (§ 3.5), the question was addressed as to whether rat caecal bacteria are able to degrade this feruloylated disaccharide or not. In an initial study, [^{3}H]arabinose was mixed *in vitro* with fresh rat caecal contents (§ 2.2). Samples were taken over a time-course and analysed for products.

3.11.1.1 Analysis of the MeOH-soluble fraction

Paper chromatography analysis of the MeOH-soluble, non-volatile products (Fig. 3.88) showed a very rapid metabolism of [^{3}H]arabinose.

To calculate the proportion of products which are volatile (volatile from acid and/or volatile from alkali) and which are non-volatile, three portions (each 100 µl) of the MeOH-soluble fraction were assayed for radioactivity (§ 2.7.1.2): one portion was assayed directly for total ^{3}H, the second portion was assayed for ^{3}H after drying from alkali and the third portion was assayed for ^{3}H after drying from acid (Fig. 3.89) and the following procedure was used:

(a) Non-volatile: total ^{3}H minus ^{3}H after drying from acid

(b) ^3H_2O and volatile ^{3}H-alcohols (e.g. [^{3}H]methanol): total ^{3}H minus ^{3}H after drying from alkali

(c) Volatile fatty ^{3}H-acids (e.g. [^{3}H]acetic acid): ^{3}H after drying from alkali minus ^{3}H after drying from acid.

Figure 3.89 (a) (assayed for total ^{3}H) indicates in comparison to figure 3.89 (b) and (c) that the major degradation products from the MeOH-soluble fraction were volatile (see also Fig. 3.97).

3.11.1.2 Analysis of MeOH-insoluble products

The MeOH-insoluble fraction from each time-point (pellet after centrifugation) was subjected to complete acid hydrolysis (§ 2.6.1) and the products were separated by PC in BAW (§ 2.5.1) (Fig. 3.90).

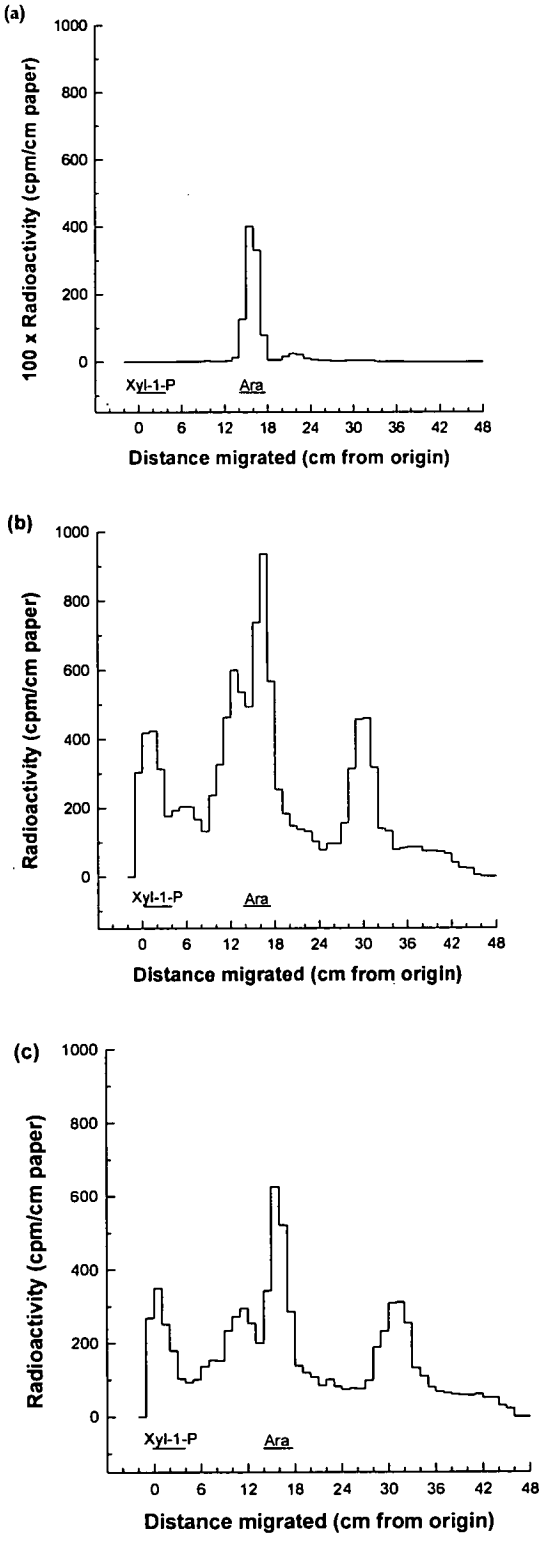


Fig. 3.88: PC in BAW (§ 2.5.1) of soluble products after treatment of [^{3}H]arabinose with rat caecal contents. Samples were taken over a time-course: (a) 0 min, (b) 5 min and (c) 120 min. Ara and Xyl-1-P served as external markers. Note the different scale used on the ordinate in (a).

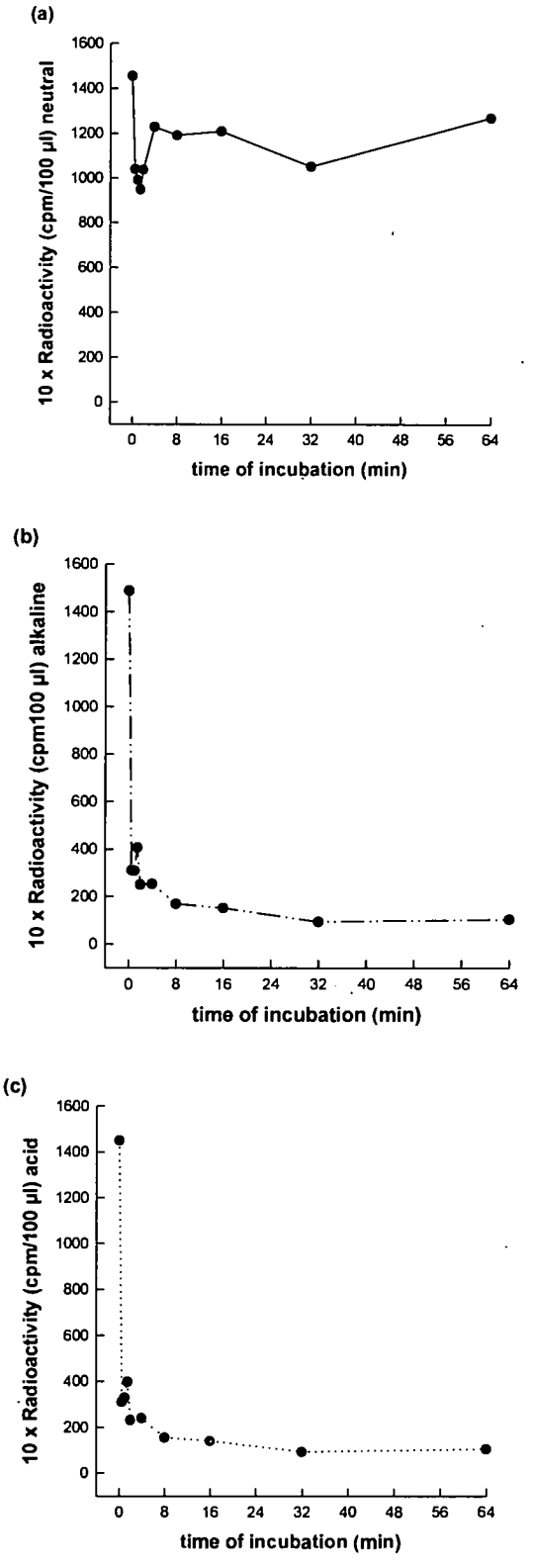


Fig. 3.89: MeOH-soluble fraction (100 µl) assayed for radioactivity: (a) assayed for total ^{3}H, (b) assayed for ^{3}H after drying from alkali and (c) assayed for ^{3}H after drying from acid.

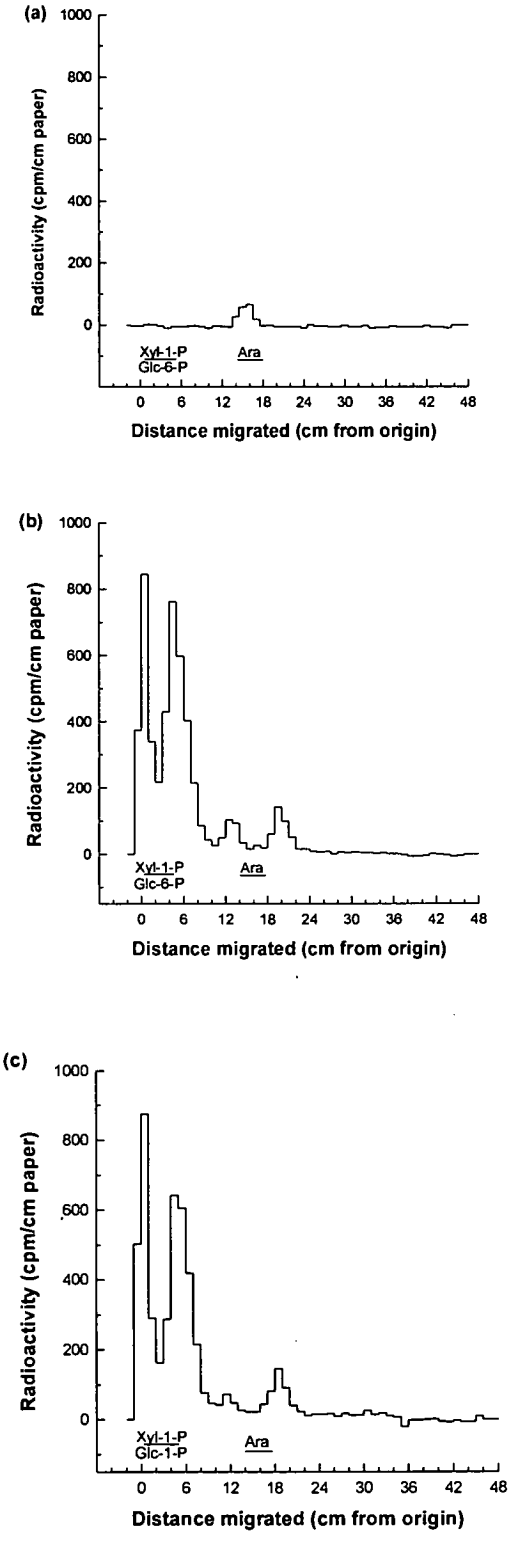


Fig. 3.90: PC in BAW (§ 2.5.1) of MeOH-insoluble products after treatment of [^{3}H]arabinose with rat caecal contents. Products from each time point were subjected to complete acid hydrolysis (§ 2.6.1): (a) 0 min, (b) 5 min and (c) 30 min. Ara, Xyl-1-P and Glc-6-P served as external markers.

3.11.2 Treatment of compound B with rat caecal bacteria

3.11.2.1 Treatment of ^{14}C-labelled compound B - analysis of the MeOH-soluble fraction

(*feruloyl*-^{14}C)-Labelled compound B was mixed with fresh rat caecal content as described in § 2.2. Samples of the MeOH-soluble fraction after fermentation were taken over a time-course and analysed for degradation products (compound A and ferulic acid: Fig. 3.91).

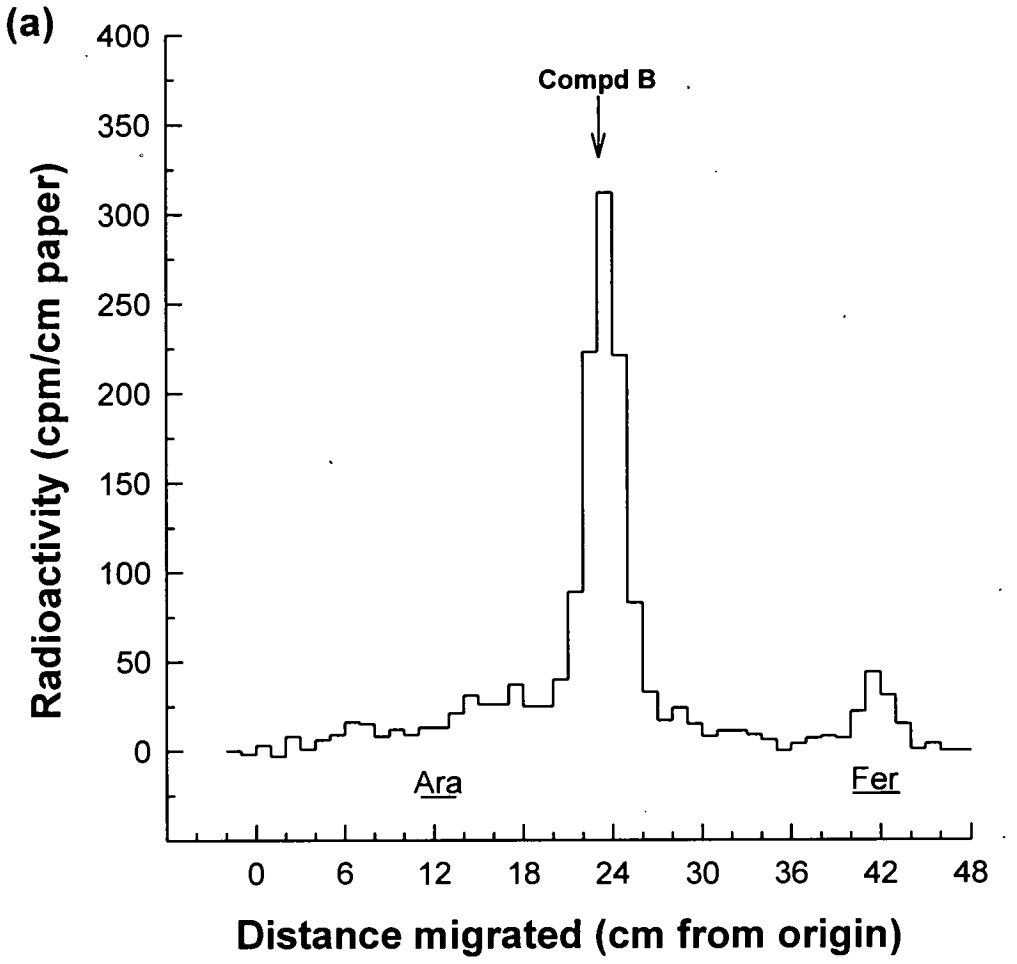


Fig. 3.91: PC in BAW (§ 2.5.1) of MeOH-Soluble products after treatment of ^{14}C-labelled compound B with rat caecal bacteria. Samples were taken over a time-course: (a) 0 min, (b) 5 min and (c) 10 min. Ara and Fer served as external markers. "Compd B" shows the approximate position of this compound (Table 3.1).

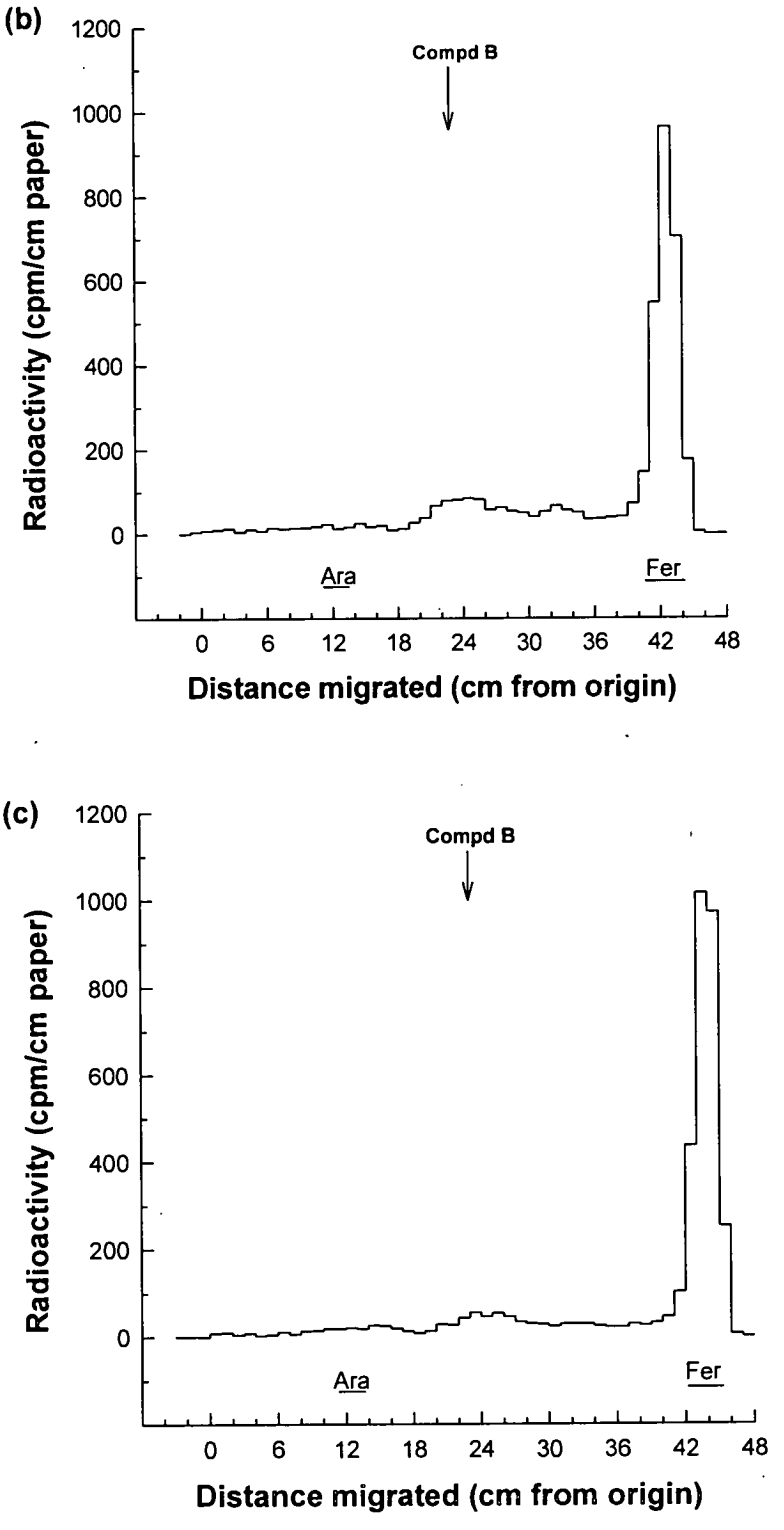


Fig. 3.91 continued.

Since almost complete degradation took place within the first 5 minutes (Fig. 3.91), the fermentation of ^{14}C-labelled compound B was carried out with shorter intervals (§ 2.2). The half-life was estimated to be ~ 0.2 min (Fig. 3.92).

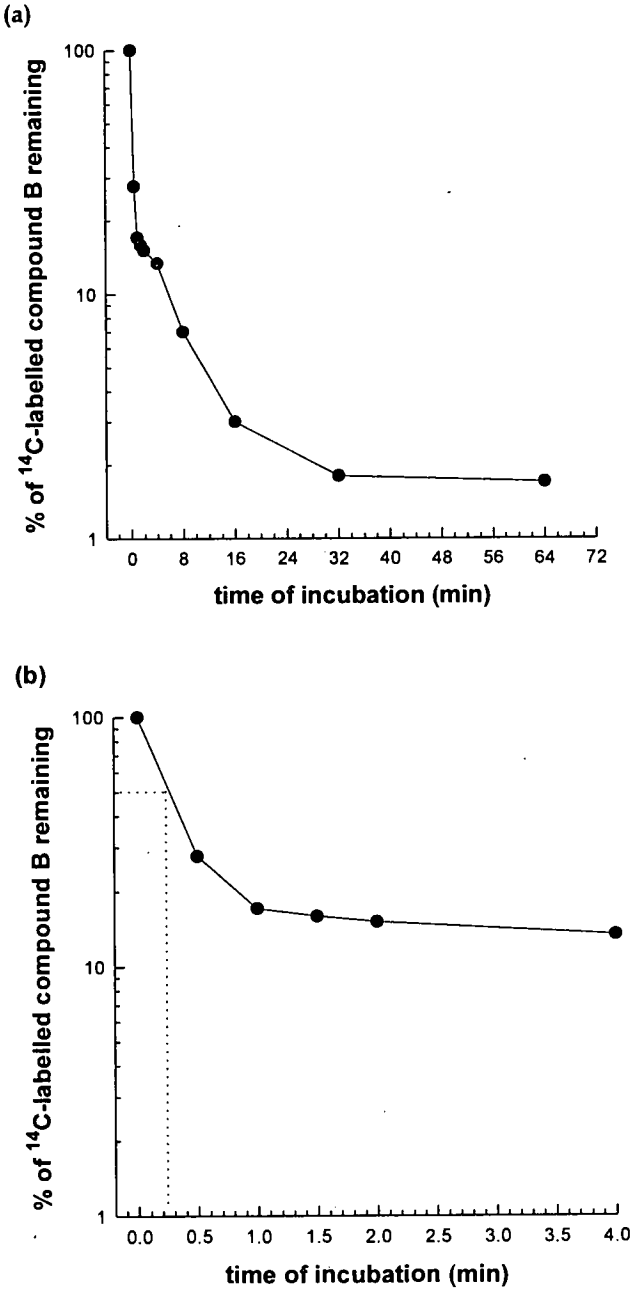


Fig. 3.92: (a) % Remaining ^{14}C-labelled compound B after fermentation with rat caecal bacteria over time. (b) Shows a section of (a) to clarify the half-life.

Routinely, approximately 5 ml rat caecal content was made up to 10 ml with medium (§ 2.2). To slow down the fermentation process, 1 ml of rat caecal content was made up to 10 ml with medium, incubated with ^{14}C-labelled compound B and analysed for degradation products. Figure 3.93 shows degradation products after 0 min and 4 min. The half-life was ~ 3 min (Fig. 3.94).

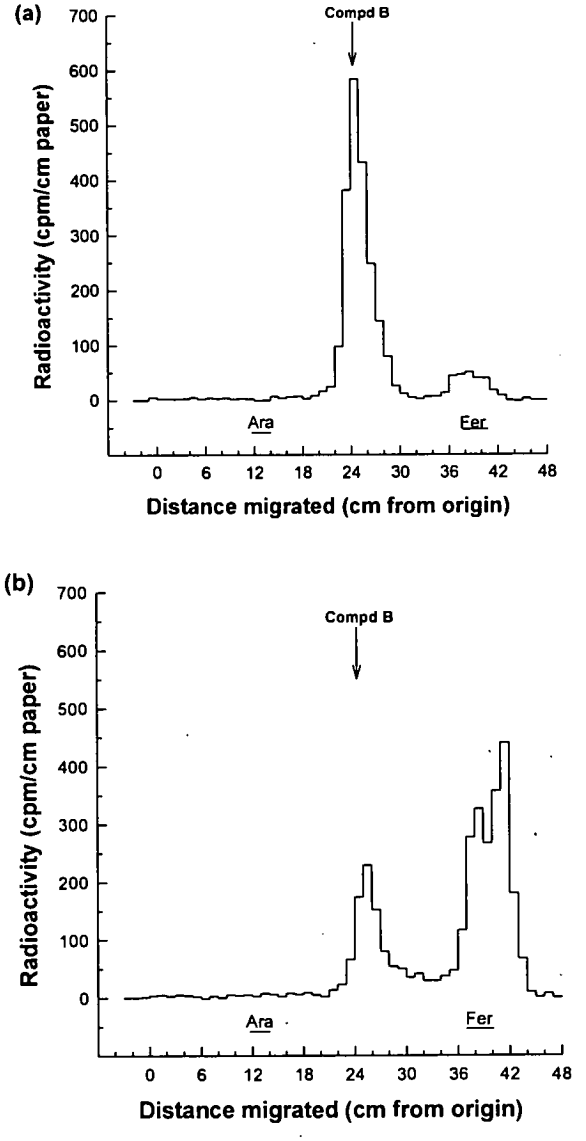


Fig. 3.93: PC in BAW (§ 2.5.1) of MeOH-Soluble products after treatment of ^{14}C-labelled compound B with diluted rat caecal bacteria. Samples were taken over a time-course and were subjected to : (a) 0 min and (b) 4 min. Ara and Fer served as external markers. "Compd B" shows the approximate position of this compound (Table 3.1).

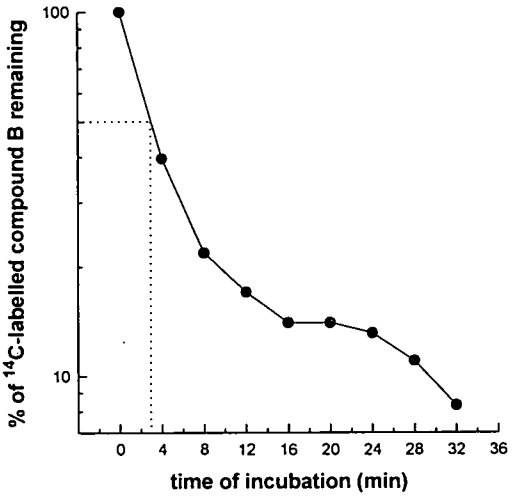


Fig. 3.94: Short-interval degradation of ^{14}C-labelled compound B with diluted rat caecal bacteria.

The half-life of ~ 3 min shows that compound B is more stable in the diluted caecal content. If there had been a linear correlation between the degradation of compound B and rat caecal bacteria concentration, a 5-fold decrease of degradation rate would have been expected. However, this is not the case (3 min rather than 1.5 min).

3.11.2.2 Treatment of ^{3}H-labelled compound B with rat caecal bacteria

(*feruloyl*-^{14}C)-Labelled compound B was mixed with fresh rat caecal content as described in § 2.2. Samples were taken over a time-course and analysed for degradation products (compound A, compound B_S, arabinose and xylose).

3.11.2.2.1 Analysis of the MeOH-soluble fraction

Since the degradation of compound B in rat caecal content took place very rapidly (Fig. 3.91), treatment of ^{3}H-labelled compound B was carried over a modified time course (shorter time interval within the first 10 min and diluted bacteria). The MeOH-soluble fraction was chromatographed. Figure 3.95 reveals as the main degradation products de-feruloylated oligosaccharides such as Ara and Ara-Xyl.

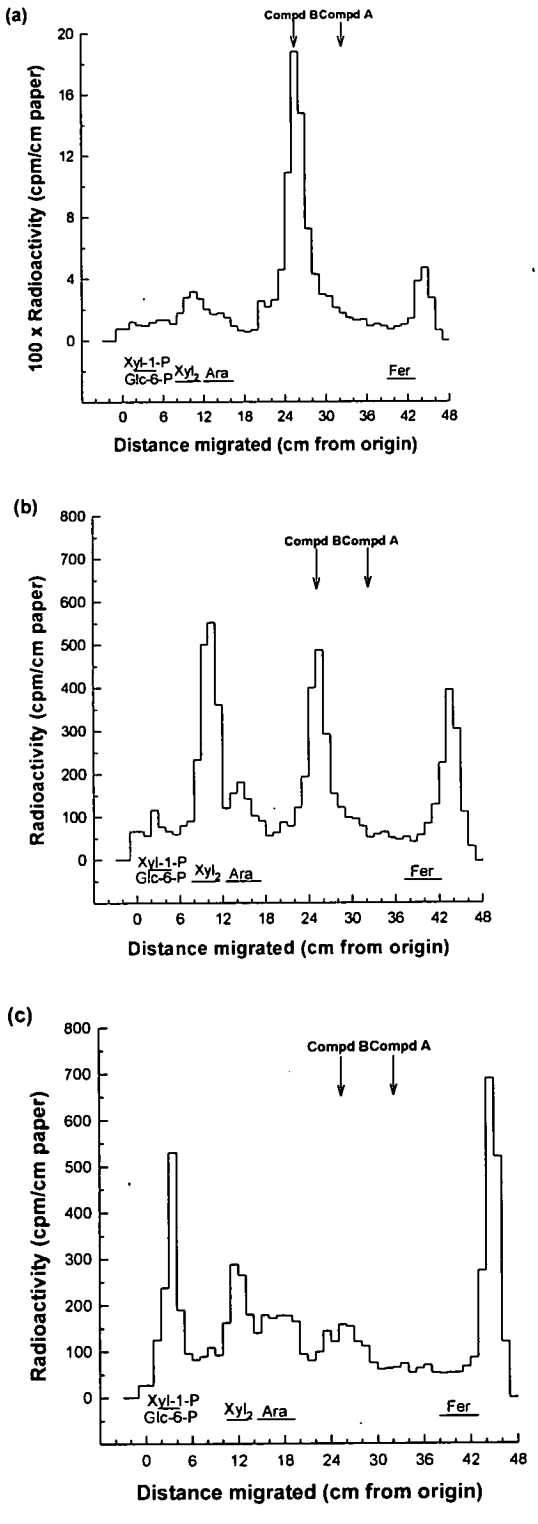


Fig. 3.95: PC in BAW (§ 2.5.1) of MeOH-Soluble products after treatment of ^{3}H-labelled compound B with diluted rat caecal contents. Samples were taken over a time-course: (a) 0 min, (b) 0.5 min and (c) 4 min. Ara, Xyl_2, Xyl-1-P, Fer and Glc-6-P served as external markers. "Compd A" and "Compd B" indicate the approximate position of these compounds (Table 3.1).

3.11.2.2.2 Volatile and non-volatile products of the MeOH-soluble fraction

The MeOH-soluble fraction was assayed for volatile and non-volatile products as described in § 3.11.1 (Fig. 3.96).

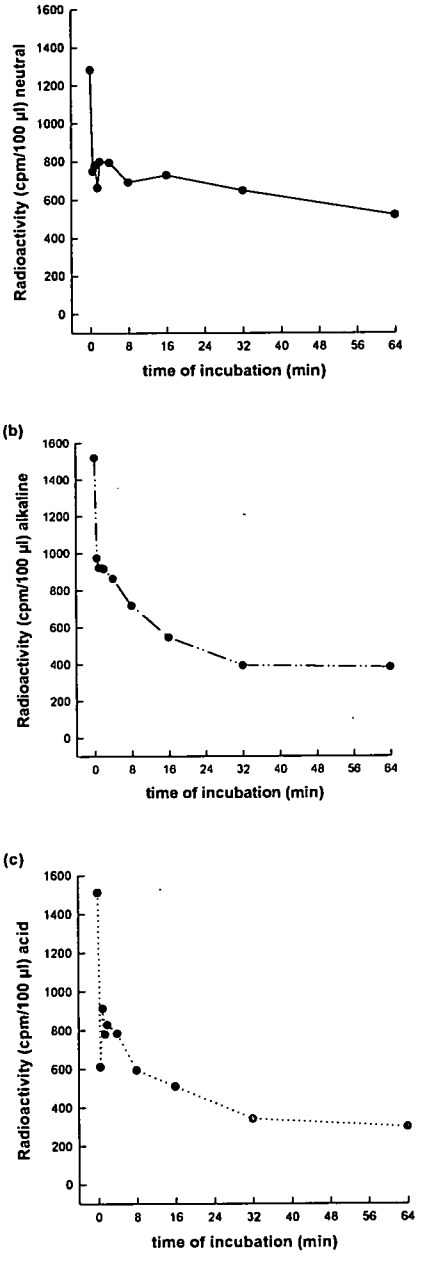


Fig. 3.96: MeOH-soluble fraction assayed for radioactivity: (a) assayed for total ^{3}H, (b) assayed for ^{3}H after drying from alkali and (c) assayed for ^{3}H after drying from acid.

3.11.2.2.3 Comparison of volatile and non-volatile products after treatment of [^{3}H]arabinose and ^{3}H-labelled compound B

Volatile and non-volatile products were calculated as described in § 3.11.1.1. Treatment of [^{3}H]arabinose with rat caecal bacteria (Fig. 3.97 (i)) released major volatile products (^3H_2O and ^{3}H-MeOH) and the major products of fermentation of ^{3}H-labelled compound B (in diluted caecal content) are non-volatile (Fig. 3.97 (ii)).

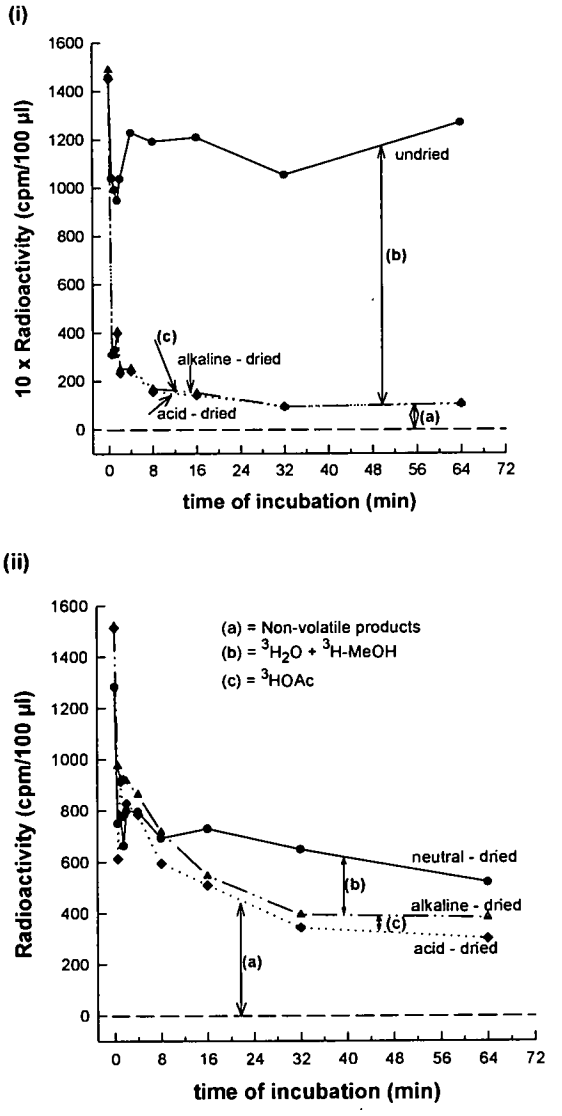


Fig. 3.97: Volatile and non-volatile products of the MeOH-soluble fraction: (i) [^{3}H]arabinose treated with rat caecal bacteria and (ii) ^{3}H-labelled compound B treated with diluted caecal content.

3.11.2.2.4 Analysis of MeOH-insoluble fraction during fermentation of ^{3}H-labelled compound B

The MeOH-insoluble fraction from each time-point was analysed after 2 M TFA hydrolysis (§ 3.11.1.3). Figure 3.98 demonstrates metabolised degradation products of compound B which co-chromatographed with e.g. arabinose and xylobiose.

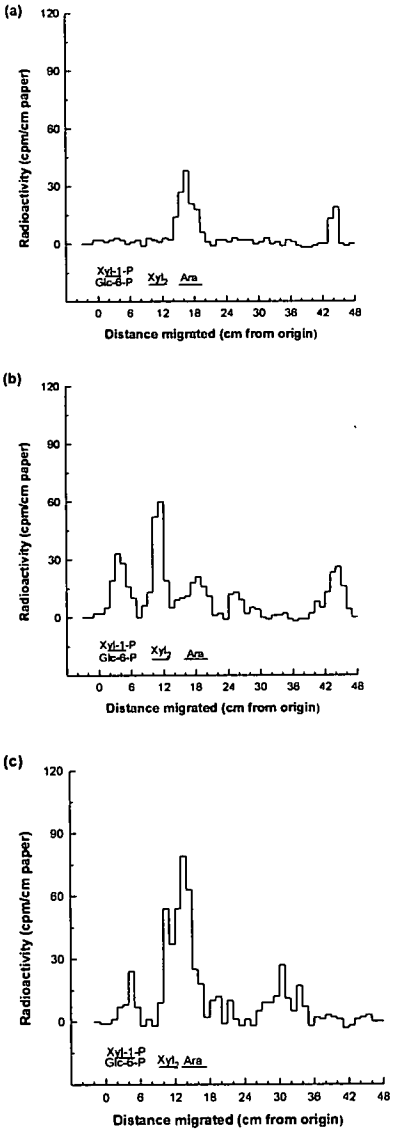


Fig. 3.98: PC in BAW (§ 2.5.1) of acid hydrolysate of MeOH-insoluble products of treatment of ^{3}H-labelled compound B with diluted rat caecal contents. Samples were taken over a time-course: (a) 0 min, (b) 0.5 min and (c) 2 min. Ara, Xyl_2, Xyl-1-P and Glc-6-P served as external markers.

3.12 Is compound B unique to *Festuca arundinacea*?

3.12.1 A taxonomic tree

To ascertain whether compound B is unique to *Festuca arundinacea* or is more widespread, a taxonomic tree was developed (modified after Clayton & Renvoize, 1989) starting with *Festuca* and monocots which are closely related to *Festuca* and progressing to others which are further apart (Fig. 3.99).

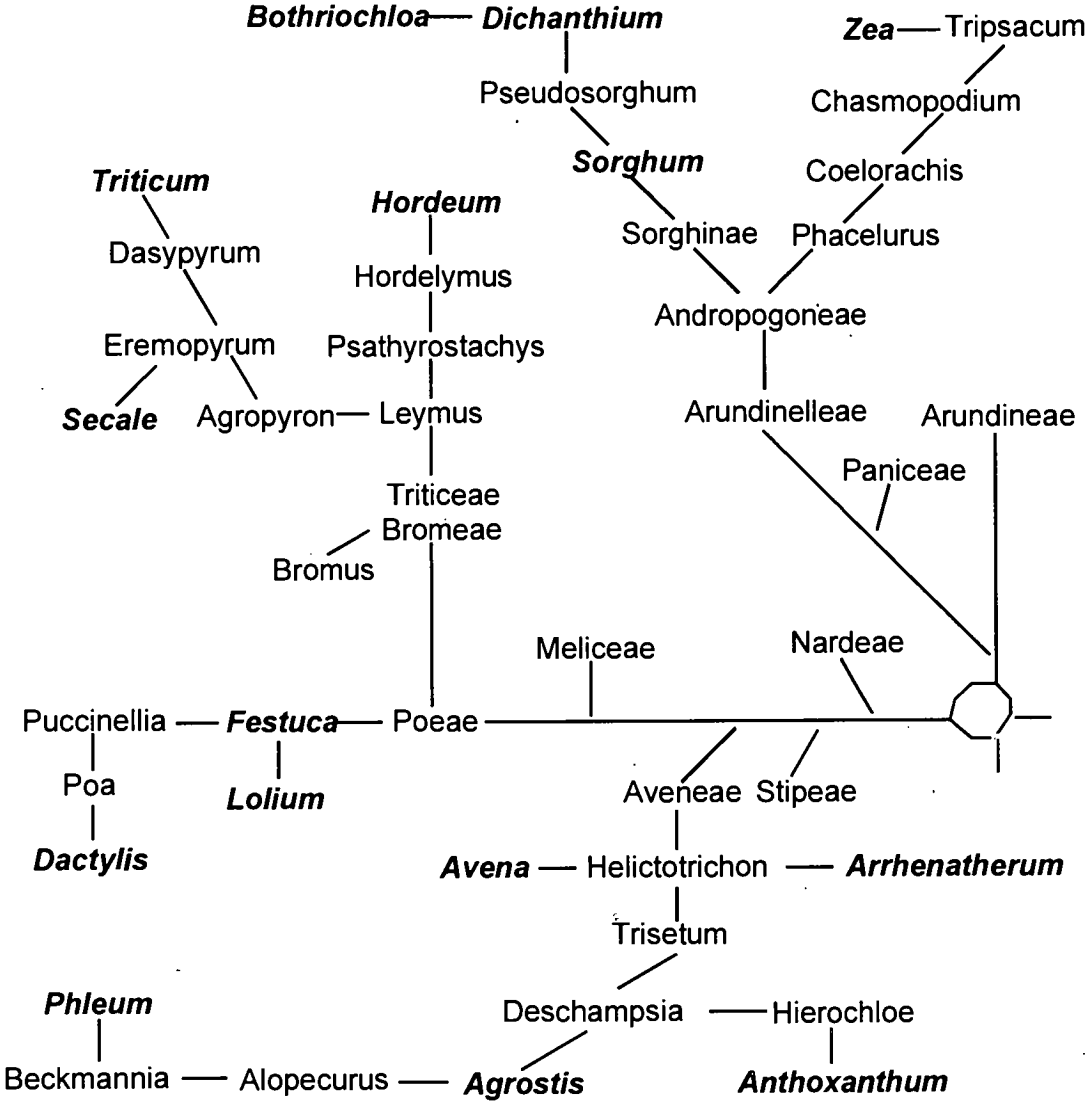


Fig. 3.99: Taxonomic tree (modified after Clayton & Renvoize, 1989). Genera shown in bold type were analysed for the presence of compound B.

3.12.2 Analysis of feruloylated compounds

3.12.2.1 Isolation of feruloylated compounds

Alcohol-insoluble residue (AIR) was prepared as described in § 2.6.9.2 from the leaves of 22 different species. A portion of the AIR was hydrolysed with mild acid (§ 2.6.1) and the hydrolysate subjected to PC in BAW (§ 2.5.1). Figure 3.100 shows the fluorescent zones (see also § 3.1) due to the feruloylated compounds from 14 different species. The results show the presence of compound B in all species investigated.

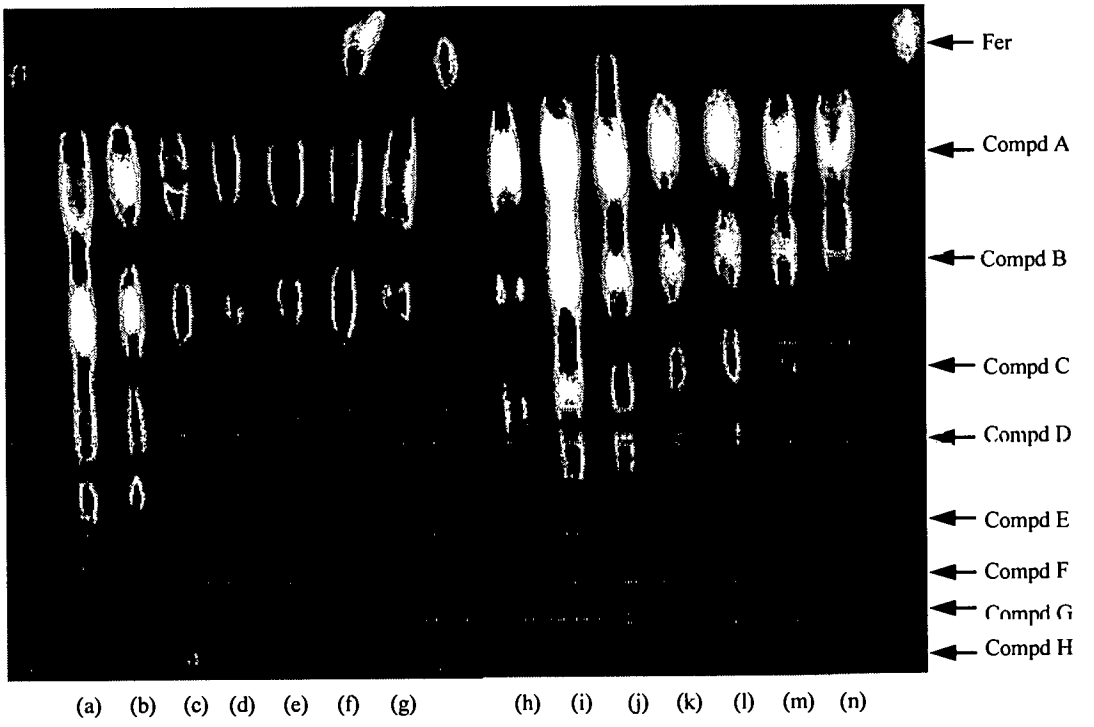


Fig. 3.100: PC in BAW (§ 2.5.1) of mild acid hydrolysis (§ 2.6.1) products of AIR of 14 different species. The chromatograms were photographed under UV light in ammonia vapour; (a) *Festuca arundinacea,* (b) *Festuca gigantea,* (c) *Festuca pratensis*, (d) *Festuca rubra*, (e) *Agrostis tenuis*, (f) *Anthoxanthum odoratum*, (g) *Arrhenatherum elatius*, (h) *Avena sativa*, (i) *Bromus sterilis*, (j) *Dactylis glomerata*, (k) *Lolium multiflorum*, (l) *Lolium perenne*, (m) *Phleum pratense* and (n) *Triticum aestivum*. *Hordeum vulgare*, *Secale sentinal*, *Sorghum verticilliflorum*, *Sorghum vulgare*, *Dichanthium sericeum*, *Bothriochloa ambigua*, *Zea mays* and *Trachycarpus fortunei* are not shown. ← Indicates the approximate position of ferulic acid and compounds A to H (origin).

3.12.2.2 Calculation of the concentration of compounds A and B

Purification of compd A and B from each species was achieved as described in § 3.1.1 and by HPLC (see also § 3.5.10.1). Table 3.18 summarises the relative amount of the two compounds obtained and their ratio.

order of ratio B/A	Name*	g AIR/ g fresh weight	presence of***		relative amount of*		ratio of compd B/A
			compd A	compd B	compd A/100mg AIR****	compd B/100mg AIR**	
12	*Festuca arundinacea*	0.612	+	+	16.84	9.92	0.59
13	*Festuca gigantea*	0.348	+	+	12.35	7.12	0.58
1	*Festuca pratensis*	0.452	+	+	6.88	14.43	2.10
11	*Festuca rubra*	0.638	+	+	8.74	5.63	0.64
5	*Lolium multiflorum*	0.546	+	+	6.94	6.97	1.00
4	*Lolium perenne*	0.256	+	+	7.36	8.17	1.11
3	*Dactylis glomerata*	0.824	+	+	17.94	21.66	1.21
6	*Bromus sterilis*	0.866	+	+	18.29	17.20	0.94
nd	*Hordeum vulgare*	0.440	+	+	nd	nd	nd
nd	*Secale sentinal*	0.472	+	+	nd	nd	nd
9	*Triticum aestivum*	0.944	+	+	8.39	6.20	0.74
14	*Avena sativa*	0.790	+	+	9.71	5.32	0.55
10	*Arrhenatherum elatius*	0.818	+	+	16.11	10.72	0.67
2	*Anthoxanthum odoratum*	0.790	+	+	19.78	24.40	1.23
8	*Agrostis tenuis*	0.594	+	+	16.86	14.70	0.87
7	*Phleum pratense*	0.720	+	+	13.77	12.09	0.88
nd	*Sorghum verticilliflorum*	0.552	+	+	nd	nd	nd
nd	*Sorghum vulgare*	0.492	+	+	nd	nd	nd
nd	*Dichanthium sericeum*	0.816	+	+	nd	nd	nd
nd	*Bothriochloa ambigua*	0.744	+	+	nd	nd	nd
nd	*Zea mays*	0.680	+	+	nd	nd	nd
nd	*Trachycarpus fortunei*	0.844	+	+	nd	nd	nd

Table 3.18: Calculation of the ratio of compound A/B per 100 mg AIR from 14 different species. *Arbitrary units calcutated by HPLC trace, ** by triangulation, ***Qualitative study by PC, ****Quantified *in silico* by integrator,*all Gramineae except *Trachycarpus fortunei* (Palmae).

The result showed the presence of compound B in all species investigated. The highest amount of compound A and B was obtained from *Anthoxanthum odoratum* (19.78 and 24.40, respectively) and the lowest amount of compound A from *Festuca pratensis* (6.88) and the lowest amount of compound B from *Avena sativa* (5.32). However, *Festuca pratensis* showed the highest ratio of compound B to compound A (2.10) and *Avena sativa* the lowest (0.55). The ratio of compound B to A in *Festuca arundinacea* was very close to that in *Avena* (0.59).

4. Discussion

This thesis has explored some aspects of feruloylated polysaccharides in the primary cell walls of *Festuca arundinacea*.

4.1 Preparation of AIR

Cultured cells of *Festuca arundinacea* incorporated ^{3}H from exogenous L-[1-^{3}H]arabinose into newly-formed polysaccharides in the form of [^{3}H]arabinose and [^{3}H]xylose residues. Incubation for 5 h gave the largest amount of ^{3}H-labelled compounds. Incubation for a much longer period (6 days under sterile conditions) gave a negligible increase in the radioactivity in wall components, suggesting that the uptake of Ara is rapid. The specific radioactivity of [^{3}H]arabinose was found to be higher than that of [^{3}H]xylose (§§ 3.5.5.1 and 3.6.2), showing that the reaction UDP-L-[^{3}H]Ara ⇔ UDP-L-[^{3}H]Xyl is not at equilibrium *in vivo*.

4.2 Release of feruloylated compounds

4.2.1 Mild acid hydrolysis

The attachment of ferulic acid to polysaccharides is widespread in the cell walls of monocot families, especially the Gramineae (Hartley & Jones,1977) and in the dicot family Chenopodiaceae (Fry, 1979).

The structures of the feruloylated polysaccharides of *Festuca arundinacea* were investigated from two different directions: fragments released after mild-acid treatment and fragments released after enzymic hydrolysis (Driselase).

The acid of choice for hydrolysis was TFA because it is volatile and for removing the acid no further step is necessary than to dry the sample *in vacuo*. For mild acid hydrolysis, 0.1 M TFA at 100°C for 1 h was used to cleave the arabinofuranose residues from the xylan backbone (pyranosides and feruloyl esters are considerably more stable to mild acid hydrolysis than sugars in furanosides - Fry, 1983). These conditions were found to achieve the highest yield of compound B. However, it turned out that compounds once released by mild acid hydrolysis could still be appreciably broken down by further treatment with 0.1 M TFA at 100°C for 1 h (3.10.2.5).

4.2.2 Detection of feruloylated material

The products released (either by acid hydrolysis or by Driselase) from fescue cell walls were separated by PC in BAW. Eight blue fluorescent zones (A to H - released after acid hydrolysis, Fig. 3.1; 1 to 6 - released after enzymic hydrolysis, Figs. 3.79 and 3.80) could be detected with UV-fluorescence properties strongly suggesting feruloyl derivatives (Fry, 1982, Kato & Nevins, 1985). The UV-absorption spectra of compounds A to H before and after alkaline hydrolysis were also consistent with feruloyl esters (Jurd, 1957; Smith & Hartley, 1983). Fluorescence characteristics of *p*-coumaryl esters (invisible at neutral or acidic pH, turning to blue during fuming with NH_3 vapour) could not be detected. The release of feruloylated material by mild acid is consistent with compounds A to H being attached via a feruloylated arabinofuranose to their parent polymer. The release of feruloylated material by enzymic hydrolysis (6 fluorescent zones, Fig. 3.79) is consistent with compounds 1 to 6.

4.2.3 Purification of compounds

Compounds A and B could be separated quite effectively. This was not true for the other compounds (C to H). The arrows in Figs. 3.1(a) and 3.79 indicate the middle of each zone. The clear identification of compounds C to H caused difficulties, because these zones were incompletely resolved in some cases (the same is true for compounds 1 to 6).

In order to separate compounds A - H (and compounds 1 to 6) from non-aromatic products, each compound was passed through an RPC column (§ 2.6.5). This, followed by PC in BEW, allowed a satisfactory purification to be achieved (Tables 3.1 and 3.16).

Within a homologous series, the slower a carbohydrate migrates (on PC) the higher is its molecular weight. Thus, compound H and compound 6 are likely to be large oligosaccharides and compound A probably a feruloylated monosaccharide.

The presence of a feruloyl group is likely to confer a high chromatographic mobility (because of its non-polar character). After cleavage of the ester bond we would expect a decrease in R_f of the carbohydrate moiety. In order to obtain the de-feruloylated ^3H-labelled sugar

moieties, the compounds were subjected to alkaline hydrolysis (§ 2.6.2). The de-feruloylated compounds showed, on average, approximately 1/3 of their previous R_f (Fig. 4.1 shows this for compounds A to F).

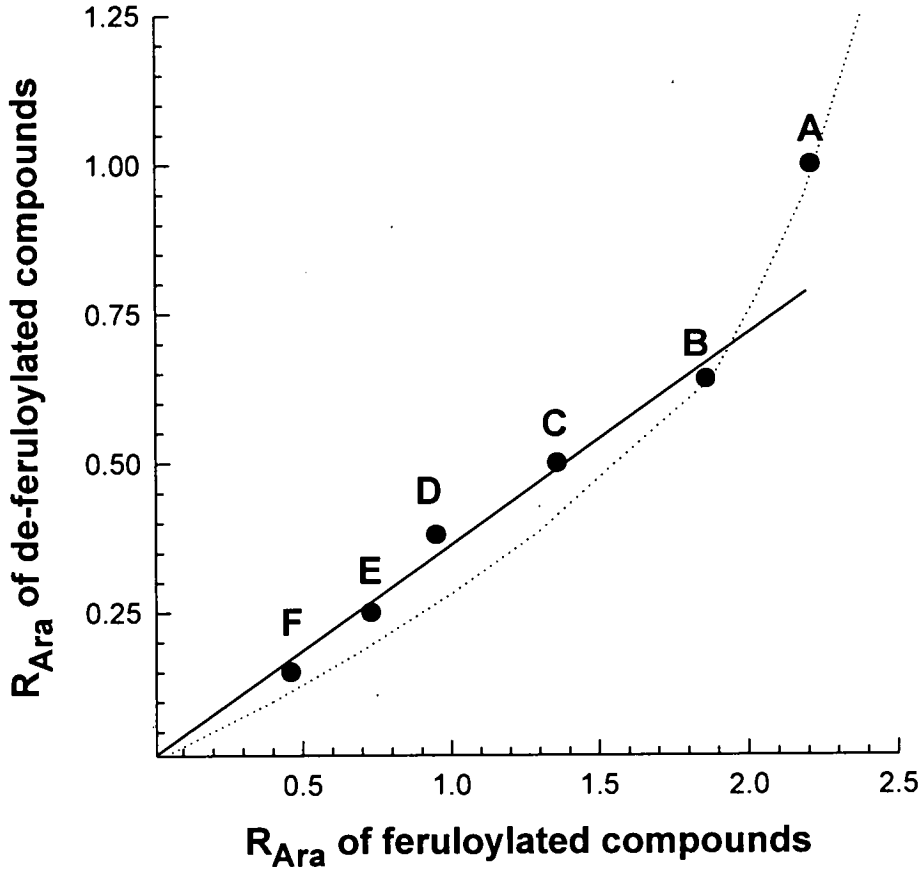


Fig. 4.1: R_{Ara} -values of feruloylated and de-feruloylated compounds. (····· ideal tendency of the curve) (2.8/2.8) = maximum.

Taken together, the evidence showed that the acid- and Driselase-released fragments were feruloyl derivatives since they showed a blue fluorescence under UV light (turning to intense blue-green under ammonia vapour), they were labile to alkaline hydrolysis (see § 4.3) and they revealed a characteristic UV absorption spectrum (the latter demonstrated for compounds A and B). It is suggested that no *p*-coumaroyl esters were present since their R_f-values are slightly higher in BEW than corresponding feruloyl esters (Fry, 1989) and they could not be detected.

4.2.4 Comparison of chromatographic data for acid- and Driselase-released fragments

Table 4.1 summarises chromatographic data for these compounds and the molar Ara : Xyl ratios after 2 M TFA hydrolysis of saponified compounds. The R_{Ara}-values for instance of compound 1 and compound B were very similar and therefore they probably had similar molecular weights (the same was true for compounds 2/C, 3/D, 5/G and 6/H). The structures presented for compounds 1 and B (Figs. 3.13 and 3.81 (a)) propose that both compounds are feruloylated disaccharides. Both compound 2 and C are believed to be feruloylated trisaccharides (Figs. 3.37 and 3.81 (b)). From the Ara : Xyl ratios, presumably compounds 1 and B are made up of 1 Ara and 1 Xyl and compounds 2 and C contain 1 Ara and 2 Xyl.

Compound	R_{Ara} (in BAW)	Molar Ara : Xyl ratios after 2 M TFA hydrolysis	Proposed DP of oligosaccharide
1	1.88	0.96	2
B	1.86	1.06	2
2	1.38	0.43	3
C	1.36	0.52	3
3	0.88	0.33	5
D	0.95	0.48	5
E	0.73	0.33	7
5	0.31	nd	nd
G	0.29	nd	nd
6	0.00 - 0.05	nd	nd
H	0.00 - 0.03	nd	nd

Table 4.1: Chromatographic data on PC in BAW obtained for feruloylated oligosaccharides released from *Festuca* AIR by acid (compounds B, C, D, G and H) and by Driselase (compounds 1, 2, 3, 5 and 6).

4.3 Alkaline hydrolysis

4.3.1 General alkaline hydrolysis

Routinely, alkaline hydrolysis was performed for 1 h. Each de-feruloylated compound (e.g. compds A_S, B_S, 1_S, 2_S...) appeared to give a single product (on the basis of PC in BAW and BEW) with the exception of compound C which gave C_S and C_S' (Fig. 3.47).

Alkaline hydrolysis, for instance of ^3H-labelled compound A, yielded a single radioactive product (compound A_S) which co-chromatographed with authentic non-radioactive arabinose (Fig. 3.8) indicating that compound A is a feruloylated monosaccharide. Compound B_S migrated much slower than arabinose (R_{Ara} = 0.27, in EPW_2), suggesting that compound B is a feruloylated oligosaccharide.

4.3.2 Test for multiple esters of compounds released by mild acid

Table 4.2 (based on figures 3.10, 3.16, 3.49, 3.56 and 3.67) shows that the half-life periods of the feruloyl residue in compounds A, B, C, D and E were all less than 15 min. Therefore, after 1 hour, saponification will have been ≥93% complete.

Compound	half-life (min) after saponification for 1 h
A	~2
B	~14
C	~8
D	~4
E	~3

Table 4.2: Half-life periods of the feruloyl residue in compounds A, B, C, D and E. Partial saponification was performed at 25°C and pH ~12.8 (compds A and B) and pH ~13.3 (compds C, D and E).

It has been reported that xylans extracted from monocots under mild conditions (dimethyl sulphoxide) contain *O*-acetyl groups (Hirst, 1962). Ishii (1991) and Azuma *et al.* (1990) found in addition to ferulate an *O*-acetyl group: an acetylated and feruloylated arabinoxylan trisaccharide derived from bamboo shoot and an acetylated and feruloylated arabinoxylan tetrasaccharide from mature sugar-cane bagasse, respectively.

To test for multiple esters (e.g. in compound A), partial saponification was carried out. If compound A, for instance, is *O*-feruloyl arabinose, we would expect to get a single radioactive product even after partial saponification. If there was another ester linkage, e.g. of an *O*-acetyl group, we would see at least two radioactive products.

The three "levels" (① to ③) in figure 4.2 would probably be resolved by PC. In ①, the sugar is shown connected to two non-polar groups (Fer and OAc) and ought to show a faster migration than in ② (the same sugar connected either to Fer or to OAc). The slowest migration should be shown by the sugar itself (level ③ without any ester bond).

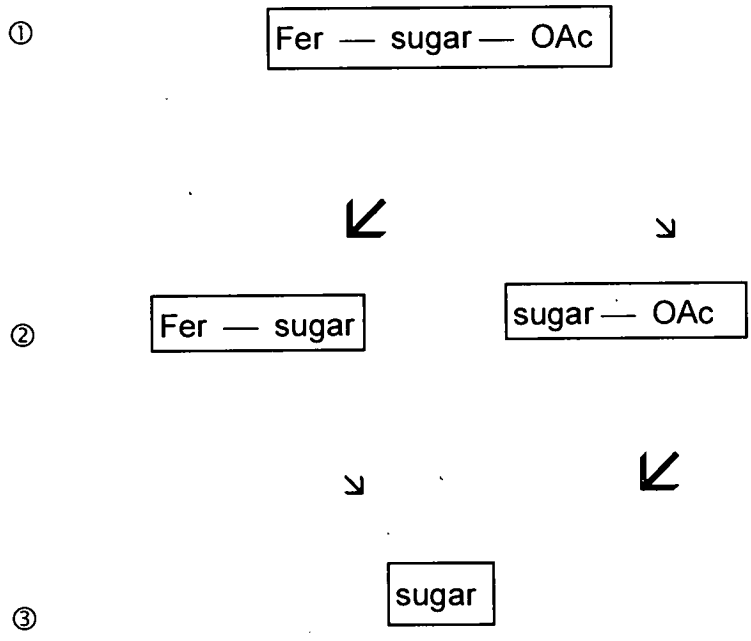


Fig. 4.2: Diagram of possible multiple esters of a sugar and their products after partial saponification.

The results obtained showed clearly that ferulate was the sole ester group present in compounds A, B and C (Figs. 3.11, 3.17 and 3.48). However, from compounds D and E (Figs. 3.57 and 3.68) at least one intermediate product was obtained: compounds D' and E', respectively, are products with lower R_{Ara}-values than the original compounds. Further alkaline hydrolysis for 1 h converted compound E' to ferulic acid and compound D' to ferulic acid and another product.

In an attempt to identify the additional ester group within compounds D and E, a suspension culture of fescue was fed with [^{14}C]acetate for 32 h (a period shown in a preliminary study to obtain the highest yield of incorporated [^{14}C]acetate). Both compound D and E became radioactive, suggesting that compounds D and E contained an *O*-acetyl group in addition to *O*-ferulate.

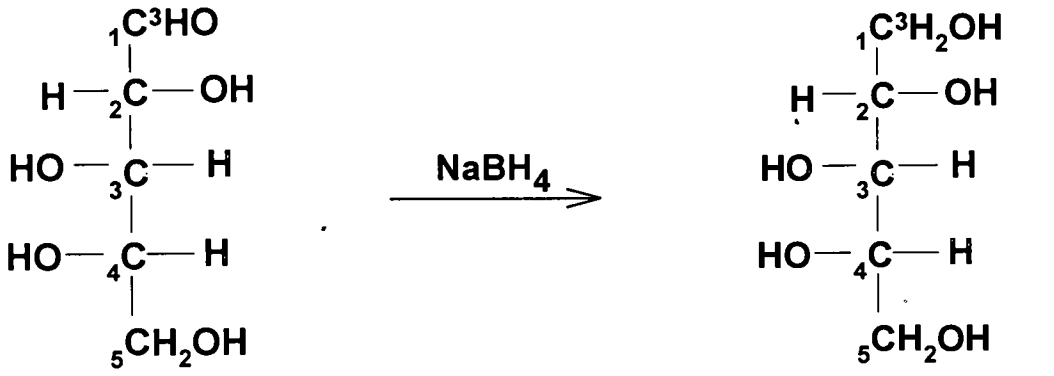
Alkaline hydrolysis will cleave the *O*-acetyl group first and then the linkage to *O*-ferulate. This behaviour is consistent with the significant shorter half-life periods of compounds D and E after saponification (~4 min and ~3 min, respectively; Table 4.2) in comparison to those of compound B (~14 min) and compound C (~8 min).

4.4 Reduction

4.4.1 General reduction of compounds released by mild acid

Compounds A to H (released by mild acid hydrolysis) had probably been connected via their arabinose residues to the parent polymer (for linkage see § 4.5) as indicated in figures 3.13 and 3.37. Mild acid hydrolysis (0.1 M TFA) would cleave this linkage and would set free compounds with arabinose at the reducing terminus. In order to test this, $NaBH_4$ was chosen as a reducing agent to reduce aldehydes to alcohols, e.g. arabinose to arabinitol (Fig. 4.3) because of its chemoselectivity (it will reduce no groups in sugars other than aldehydes and ketones).

The mechanism of the sodium borohydride reduction indicates that 1 mol $NaBH_4$ reduces 4 mol sugar (Beyer & Walter, 1991, Organikum, 1986) (Fig. 4.4).



L-[3H]Arabinose

L-[1-3H]Arabinitol
(= L-[5-3H]Lyxitol)

Fig. 4.3: Reduction of L-arabinose to L-arabinitol with $NaBH_4$.

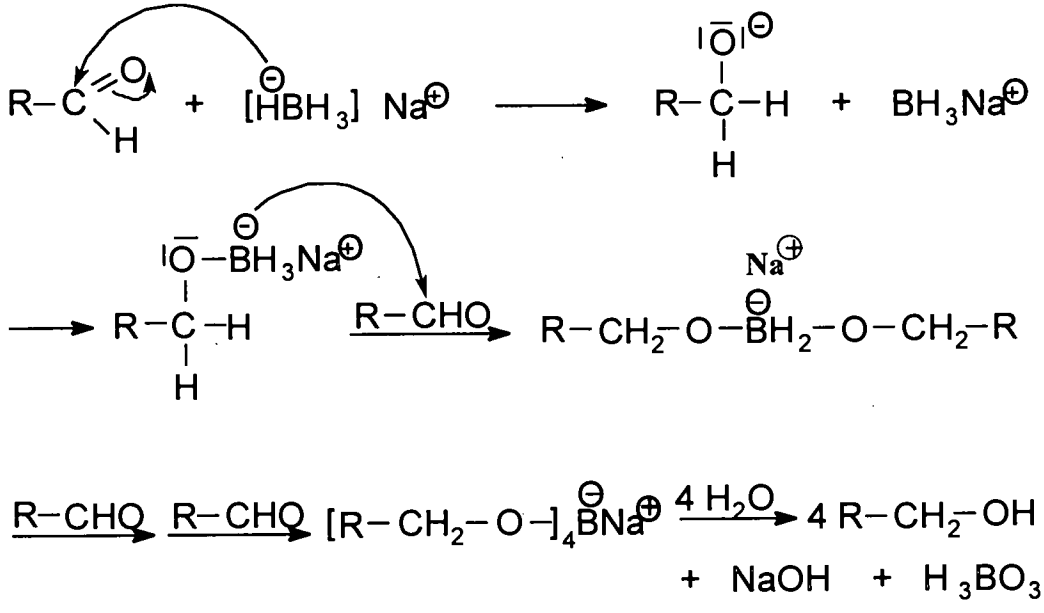


Fig. 4.4: Mechanism of the sodium borphydride reduction (modified after Beyer & Walter, 1991 & Organikum, 1986).

The reduction with sodium borohydride was carried out in the initial experiments for 4 h in the dark. Under these conditions, compound A_S yielded arabinitol as the sole radioactive product. This helps to confirm the

suggestion, from chromatography data, that compound A_S is arabinose. Arabinose has been found in plant cell walls only as the L-enantiomer (Wilkie, 1979). It is unlikely that exogenous L-[1-^{3}H]arabinose would retain radioactivity during possible conversion to the D-isomer. Therefore, compound A is concluded to be *O*-feruloyl-L-arabinose.

However, reduction of compound B_S (a disaccharide of arabinose and xylose) (and its higher homologues and Driselase released products) was not successful after incubation with $NaBH_4$ for 4 h. For instance, migration of putative compound B_S-ol (in an initial experiment; data not shown) was similar to that of compound B_S in figure 3.14. It can be presumed that the reduction of compound B_S to compound B_S-ol was not as intended.

Smith & Hartley (1983) found an unusual stability of carbohydrates against reduction with sodium borohydride when a (1→2)-linkage is involved. This was taken as a first indication of the linkage within compound B_S. Therefore it was necessary to optimise the conditions for the $NaBH_4$-reduction.

Complete acid hydrolysis of a freshly prepared portion of B_S-ol (incubation with $NaBH_4$ for 12 h with repetition) finally adduced clear evidence that arabinose is the reducing terminus of compound B. This could be shown in two different systems:

(a) Separation by PC in BAB (Fig. 3.21): the first peak of ^{3}H represented xylose. Since the migration of arabinose is significantly slower than arabinitol and xylitol, the second peak was presumed to be arabinitol.

(b) Separation by PE in borate buffer at pH 9.4 (Fig. 3.22): a satisfactory separation of xylose, xylitol and arabinitol was achieved. After staining the internal markers (§ 2.8.2), arabinitol corresponded exactly with the first radioactive product and xylose exactly with the second. This result clearly indicates a reducing terminus arabinose moiety and a non-reducing terminus xylose moiety.

A reducing terminus arabinose could also be demonstrated for compound C_S. Reduction of this trisaccharide (2 x $NaBH_4$-treatment for 12 h) followed by complete acid hydrolysis released arabinitol and xylose (Fig. 3.41).

$NaBH_4$-reduction (2 x for 12 h) of compound 2_S followed by complete acid hydrolysis, however revealed a reducing terminus xylose (§ 3.10.2.3, Fig. 3.83). Since this compound was released by Driselase, it is believed to contain part of the xylan backbone (see Fig. 3.81 for the structure).

4.4.2 Investigation of the glycosidic linkage within compound B

Some neutral carbohydrates show the ability to bind to anions e.g. molybdate (Henderson *et al.,* 1899). Weigel (1963) demonstrated that the ability of a sugar to bind to molybdate is due to a cis-cis-1,2,3-triol system. By this binding, the carbohydrates acquire a negative charge and, therefore, electrophoretic mobility. It was found that the binding to alditols (e.g. arabinitol) occurs much better than to monosaccharides (e.g. arabinose). Since the molybdate-binding and mobility requires 3 correctly orientated hydroxyl groups, the blocking of a hydroxyl group in the arabinitol shows different effects on the mobility, depending on which hydroxyl group is involved (Fry, 1988). Arabinitol lacking a free hydroxyl group at position *O*-2 (2-deoxyarabinitol) is essentially immobile (when mobility is corrected for electro-endo-osmosis), whereas substitution at position *O*-5 still allows a high mobility (Weigel, 1963, Fry, 1983). Weigel has claimed that galactosyl-(1→3)-arabinitol is immobile, whereas Fry (1982) claimed that arabinosyl-(1→3)-arabinitol is moderately mobile. In contrast, I found that galactosyl-(1→3)-arabinitol had a very slight but measurable mobility (Fig. 3.23).

It was important to know the stoichiometry of sugar reduction by sodium borohydride in order to plan the reduction of 2-deoxyribose and galactosyl-(1→3)-arabinose to 2-deoxyarabinitol and galactosyl-(1→3)-arabinitol, respectively, for use as markers in paper electrophoresis (§ 3.5.7.1). In order to verify that the reduction had been successful, the standards (2-deoxyribose, 2-deoxyarabinitol, galactosyl-(1→3)-arabinose, galactosyl-(1→3)-arabinitol, methyl α-xyloside, methyl β-xyloside, methyl α-galactoside and methyl β-galactoside; for formulae see appendix) were subjected to PE in molybdate buffer. The reduction of the markers with $NaBH_4$ had to be repeated several times until suitable conditions were found.

The same was true for finding an appropriate electrophoresis buffer. Different buffers, at different pH-values and over different run times, were tested. After nearly 30 experiments it was found that molybdate buffer at pH 3.0 and 2000 V for 5 h gave the best separation of the markers: 2-substituted marker sugars were found to be immobile whereas 3-substituted marker sugars showed slight but definite mobility.

Radioactive compound B_S-ol co-migrated with the internal marker 2-deoxyarabinitol, probably purely by electro-endo-osmosis and considerably

slower than galactosyl-(1→3)-arabinitol. This result is consistent with *O*-2 rather than with *O*-3 xylosylation of arabinose in compound B.

4.5 Smith degradation

Sodium periodate will cut the linkage between diols (as indicated in Fig. 4.5 for a possible (1→2)- and (1→3)-linkage of the disaccharide within compound B_S-ol; the reaction is not stereospecific) and $NaIO_4$ itself will be reduced to sodium iodate ($NaIO_3$).

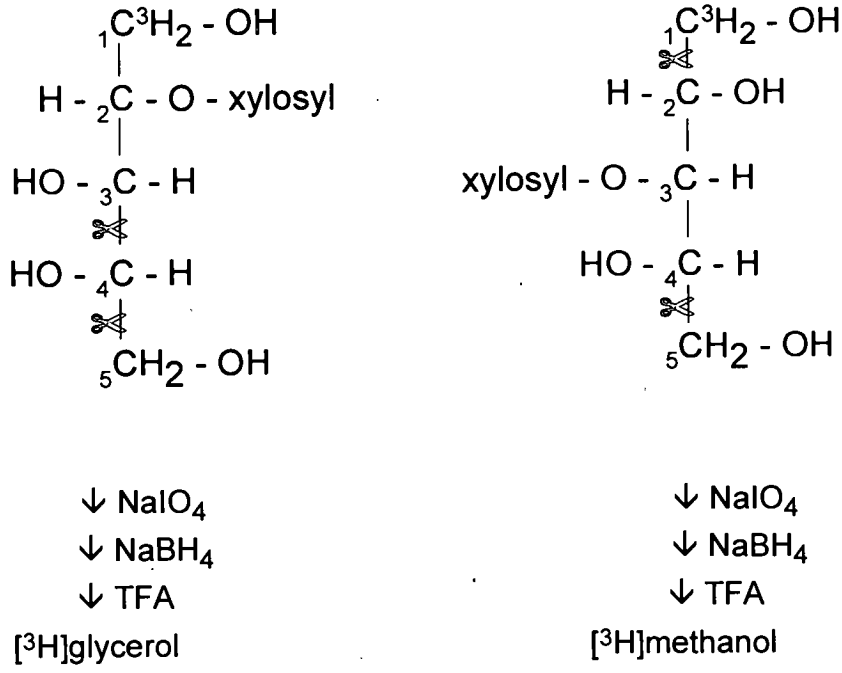


Fig. 4.5: Two possible structures of compound B_S-ol: xylose connected to *O*-2 of arabinose (left) and xylose connected to *O*-3 (right). Potential cleavages with $NaIO_4$ followed by treatment with $NaBH_4$ and TFA in reduced compound B_S-ol are indicated.

If xylose (e.g. in compound B_S-ol) is connected to *O*-2 of L-[1-^{3}H]arabinitol, $NaIO_4$-, $NaBH_4$- and TFA-treatment would result in radio-labelled glycerol, and if connected to *O*-3 of arabinitol, in a volatile product (methanol) (for the mechanism of the Smith degradation see figure 4.6).

For the $NaIO_4$-oxidation, $NaBH_4$-reduction and TFA-treatment, the conditions also had to be optimised and, therefore, this experiment was

repeated several times with modifications until the version reported was arrived at.

The radioactive moiety of compound B_S-ol after $NaIO_4$-, $NaBH_4$- and TFA-treatment co-chromatographed with authentic non-radioactive glycerol (Fig. 3.25).

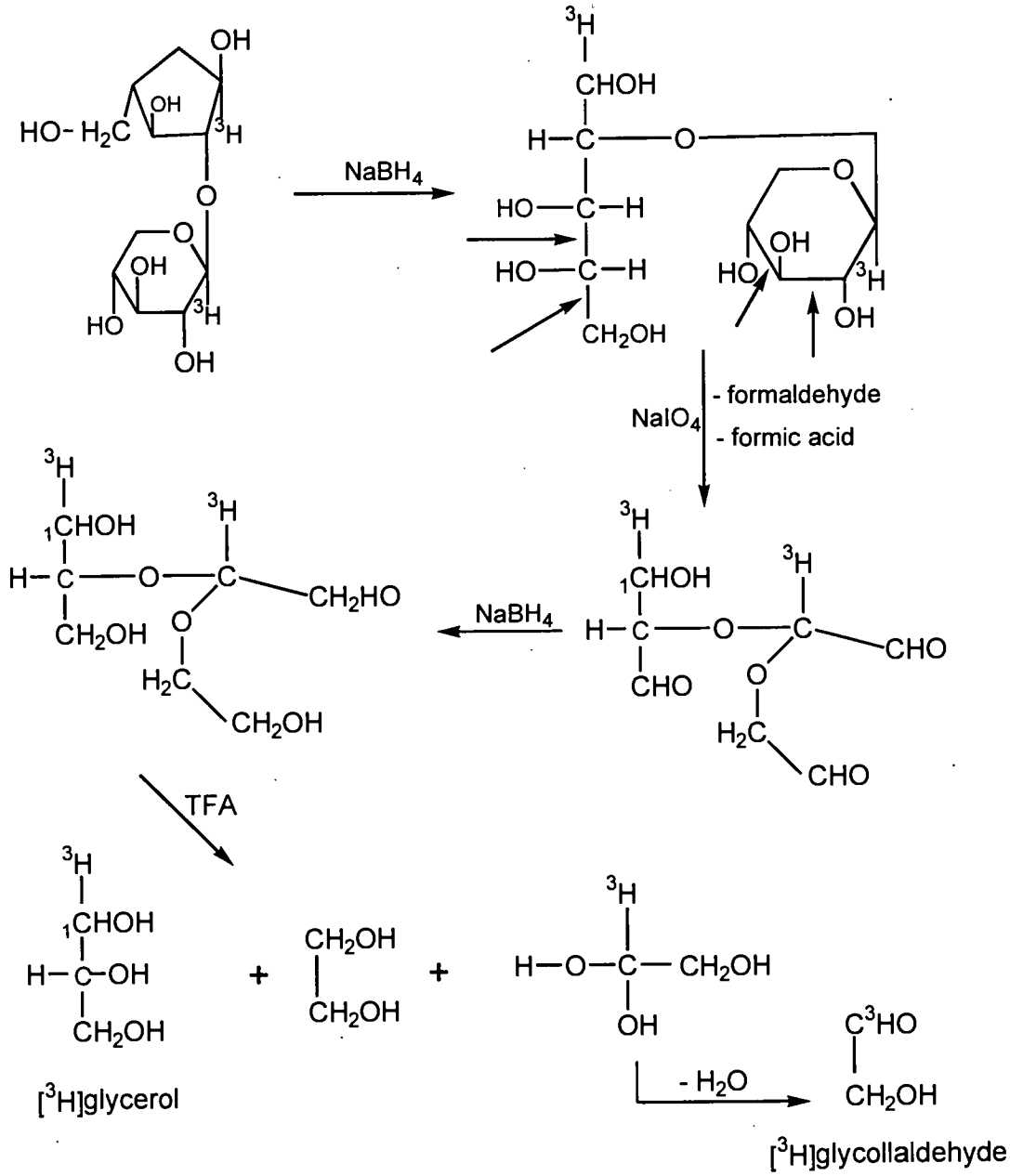


Fig. 4.6: Mechanism of the sodium periodate oxidation of (1,1'-^{3}H)-labelled compound B_S followed by TFA-hydrolysis (modified after Whistler & BeMiller, 1958). The arrows indicate possible cleavages.

Together, the data obtained from paper electrophoresis and Smith degradation gave evidence for a (1→2)-linkage of xylose to arabinose. The linkage was further supported by NMR spectroscopy and methylation analysis.

Prior to acid hydrolysis, each of compounds A to H had probably been connected via (1→3)-linkage to a β-(1→4)-D-xylopyranan backbone. This would accord with previous studies of graminaceous arbinoxylans (Woolard, Rathbone & Novellie, 1976, Smith & Hartley, 1983, Waldron & Brett, 1983). The Ara_f→Xyl_p-linkage has been reported to be in the α-configuration (Shibuya & Misaki, 1978 and Wood & MaCrae, 1986, Wilkie, 1979, Kato & Nevins, 1985), although this question has not been addressed in the present work. The configuration of the Ara_f-linkage is controversial in the literature. It has been reported that enzymic digestion (with *Oxyporus* "cellulase") will release 2-*O*-[5-*O*-(*E*)-feruloyl-β-L-arabinofuranosyl]-D-xylose from cell walls of wheat bran (Smith & Hartley, 1983). However, a β-configuration for an Ara_f residue would be highly unusual in a xylan-based polymer; β-L-Ara_f residues seem to occur mainly in the glycoprotein, extensin. Kato & Nevins (1985) found an α-L-linkage in feruloylated compounds derived from *Zea* shoot cell walls by oxalic acid followed by treatment with Driselase, connected to *O*-3 of the parent polymer: 3-*O*-[5-*O*-feruloyl-α-L-arabinofuranosyl]-β-D-xylopyranosyl-(1→4)-D-xylose (FAXX). Hartley himself (with Mueller-Harvey, Harris & Curzon, 1986) came to the same conclusion (for FAXX) by investigating the structure of feruloylated compounds derived from barley straw cell walls by *Oxyporus* "cellulase".

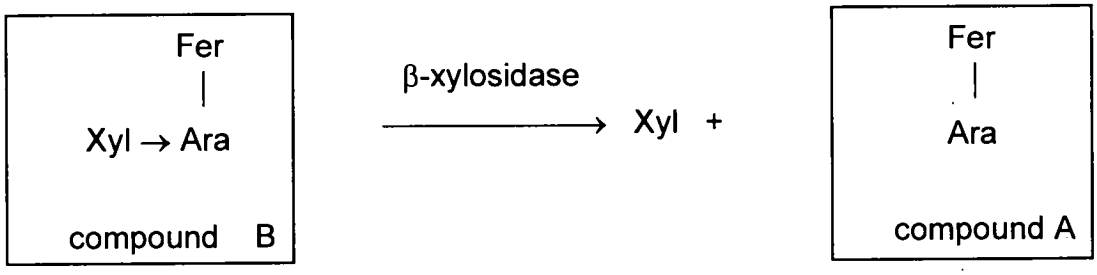
To distinguish between a (1→2)- and a (1→3)-linkage from Ara_f to Xyl_p (of the xylan backbone), $NaIO_4$-oxidation was carried out with a portion of compound 3. If the 2-*O*-xylosylated arabinose residue is linked to *O*-2' of a [1,1'-^{3}H]xylobiose group (derived from the backbone), $NaIO_4$-oxidation (without $NaBH_4$ reduction) followed by TFA-hydrolysis will yield [^{3}H]formaldehyde and [^{3}H]glyceraldehyde (from the xylobiose) and [^{3}H]arabinose. However, if the 2-*O*-xylosylated arabinose residue is linked to *O*-3' of a [1,1'-^{3}H]xylobiose group, $NaIO_4$-oxidation followed by TFA-hydrolysis will yield [^{3}H]formaldehyde and [^{3}H]xylose (from the xylobiose) and [^{3}H]arabinose. The observed products were [^{3}H]Ara : [^{3}H]Xyl (1 : 2, mol/mol), strongly supporting a (1→3)-linkage of the Ara to the xylobiose. The second molecule of [^{3}H]Xyl generated could have arisen from the Xyl

residue attached to *O*-2 of the Ara if this Xyl residue were itself substituted (e.g. with another sugar residue or an *O*-acetyl group) at its *O*-3.

It could be shown that compound C is a feruloylated trisaccharide, made of a reducing terminus arabinose and probably 2 xylose residues: compound B plus an additional xylose (see also § 4.7). Modified smith degradation (of compound C_S-ol after $NaIO_4$-oxidation, TFA hydrolysis and again $NaBH_4$-reduction) gave two major products: glycerol (from the reducing terminus arabinose) and xylitol. Since xylitol was released, a (1→3)-linkage of the additional xylose to the xylose residue of compound B can be deduced (Fig. 3.37).

4.6 Configuration of the (1→2)-linkage in compound B_S

In figure 3.13 it was suggested that there is a β-configuration of the (1→2)-linkage in compound B_S. This was investigated by enzymic digestion with β-xylosidase:



The hydrolysis was performed over a time course in order to see intermediate products. Compound A and xylose were expected (see above), but compound A was unstable so it can be presumed that an esterase was present. Therefore compound B_S and arabinose would be released as well. The predicted products (Fig. 4.7) of compound B after digestion with β-xylosidase (containing an esterase) were all observed (Fig. 3.26), except that Ara and Xyl were not resolved in the system used (PC in BAW). In addition, evidence of a β-configuration is ambiguous since the commercially available enzyme solution (Sigma) contained a high proportion of α-xylosidase (approximately 26% of xylose was released from isoprimeverose, Fig. 3.27). Subsequent purification of the enzyme solution (§ 2.6.3.2.1) was not

successful (purity was tested in different assays with a variety of substrates). A similar result was found with β-xylosidase partially purified from cucumber seeds (§ 2.6.3.2.1). The β-configuration of the (1→2)-linkage in compound B_S was, however, supported by NMR spectroscopy.

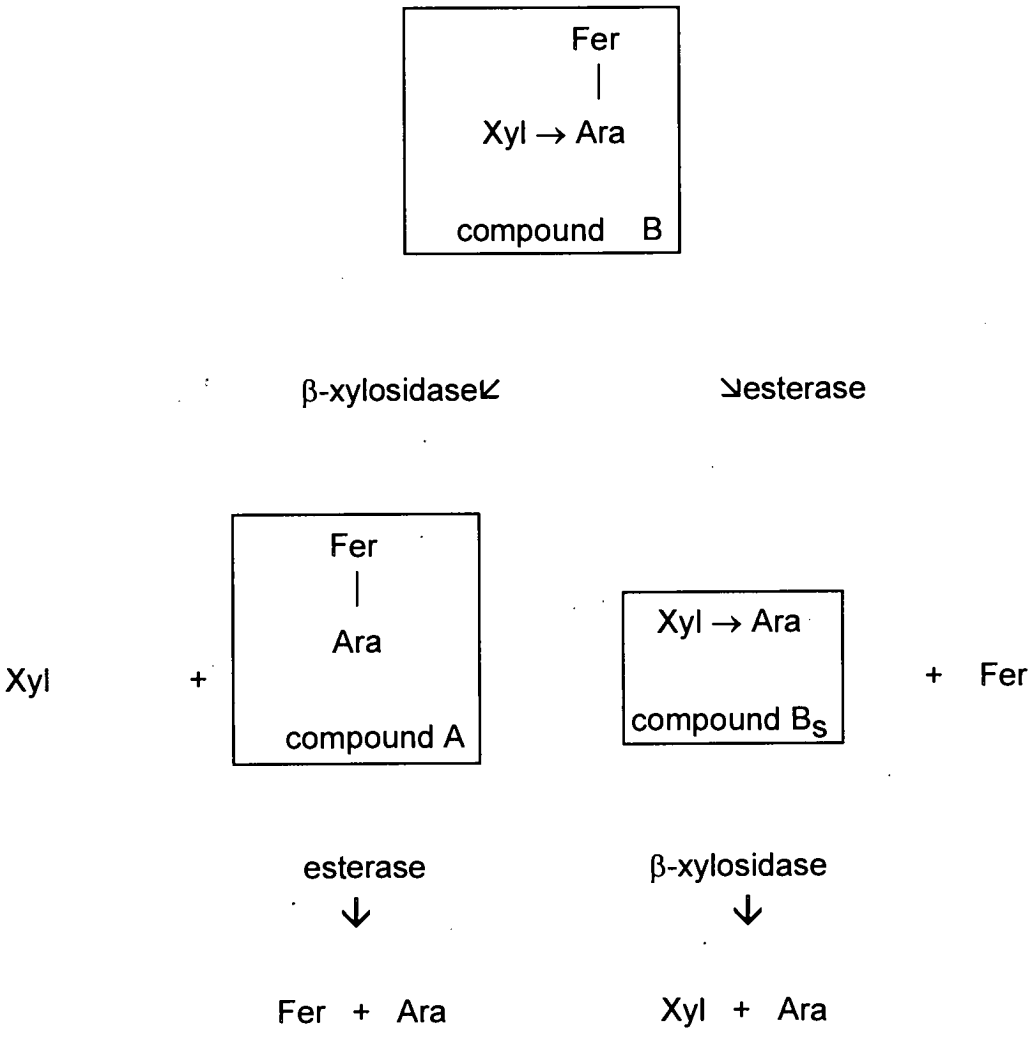


Fig. 4.7: Predicted digestion of compound B with β-xylosidase containing an esterase. Compound B would be converted to compound A + xylose by a β-xylosidase or to compound Bs + ferulic acid by an esterase. In the next step compound A and compound Bs would be transformed by an esterase and β-xylosidase, respectively, to arabinose, xylose and ferulic acid.

4.7 Driselase-treatment of compounds released after mild acid hydrolysis

An initial aim of the thesis presented was to find why *Festuca* feruloyl-arabinoxylan cannot be digested by Driselase to small fragments such as FAX and FAXX to any great extent (in comparison with e.g. maize; see figure 3.78). Prolonged treatment of compound B with Driselase revealed (§ 3.6.5.1) negligible hydrolysis of compound B.

It could be demonstrated that compound B is a component part of compounds C to H and thus responsible for the "indigestibility" of the feruloyl-arabinoxylan of fescue. This would suggest that compound B is considerable less abundant in other genera (see §§ 3.12 and 4.11).

The evidence that compound B is a part of compounds C to E was their conversion to compound B by Driselase (§§ 3.6.5.3, 3.7.3.2 and 3.8.3.2). Compound C broke down completely within 48 h, but this was not true for compounds D and E. For compound D it could be demonstrated that even after 144 h incubation with Driselase there was still some starting material (compound D) left. A reason for this might be the presence of an additional ester group in compounds D and E (Figs. 3.57 and 3.68), which causes more steric hindrance. The additional ester is believed to be *O*-acetate (§ 3.7.5).

As indicated, Driselase hydrolysis of compound C released compound B and an additional sugar. By GPC on Bio-Gel P-2 (§ 3.6.4) it could be shown that compound C_S is a trisaccharide. The data obtained after reduction of this trisaccharide followed by complete acid hydrolysis together with the data of complete acid hydrolysis of original compound C revealed an Ara : Xyl ratio of ~ 1 : 2, indicating two xylose residues present in compound C. However, the presence of xylose (in addition to compound B) after Driselase-hydrolysis could not be demonstrated clearly. A plausible explanation would be to predict a non-labelled sugar (e.g. galactose) linked to *O*-3 of the xylose residue of compound B. However, evidence for the presence of xylose as the additional sugar in compound C would mean a novel compound. The feruloylated disaccharide itself (compound B) has only been described very recently (Saulnier *et al.*, 1995) from maize bran. Himmelsbach *et al.* (1994) characterised a feruloylated tetrasaccharide with a (1→2)-linkage involved from shoots of *Cynodon dactylon*.

4.8 Position of the ester linkage

It appears that there is a significant difference between monocots and dicots in terms of the specificity of the ferulic ester linkage (Kato & Nevins, 1985). Fry (1982) identified feruloylation at position *O*-6 of galactose and at position *O*-3 of arabinose in suspension cultured spinach cells. However, ferulic acid is found to be attached to position *O*-5 of L-arabinofuranosyl side chains of the arabinoxylans of monocots (Smith & Hartley, 1983, Kato & Nevins, 1985, Nevins & Kato, 1985, MacKenzie *et al.*, 1987, Hatfield *et al.*, 1991 & Ishii, 1991). The chromatographic data presented (§§ 3.4.4 and 3.5.9) (with authentic 5-*O*-feruloyl-L-arabinose (from maize) as an internal marker) show clearly that ferulic acid is esterified to *O*-5 of arabinose in compound A. It could also be demonstrated (by PC in different solvents and by PE in borate buffer) that the feruloyl group of compound B is linked to *O*-5 of arabinose.

4.9 *In-vivo* biosynthesis of compound B

Experiments were conducted to find out at which stage the *in-vivo* feruloylation of the arabinose takes place: before or after a xylosyl residue is attached (Fig. 4.8). The polymer-bound [^{3}H]arabinose residues started to be *O*-feruloylated immediately after their incorporation into the polysaccharide (<5 min lag). It is concluded that the feruloylation is intraprotoplasmic. Thus, the data in this thesis confirms the work of Myton (1993) but is in contradiction to the results of Yamamoto & Towers (1985), who suggested that there may be a modification to the wall bound polysaccharides, for instance *in muro* feruloylation. When [^{14}C]ferulate was fed, the Fer-Ara and Fer-(Xyl)-Ara groups (as revealed by compounds A and B, respectively) both started to become ^{14}C-labelled with a lag of <1 min, suggesting that, to produce Fer-(Xyl)-Ara, the Ara is first xylosylated and then feruloylated.

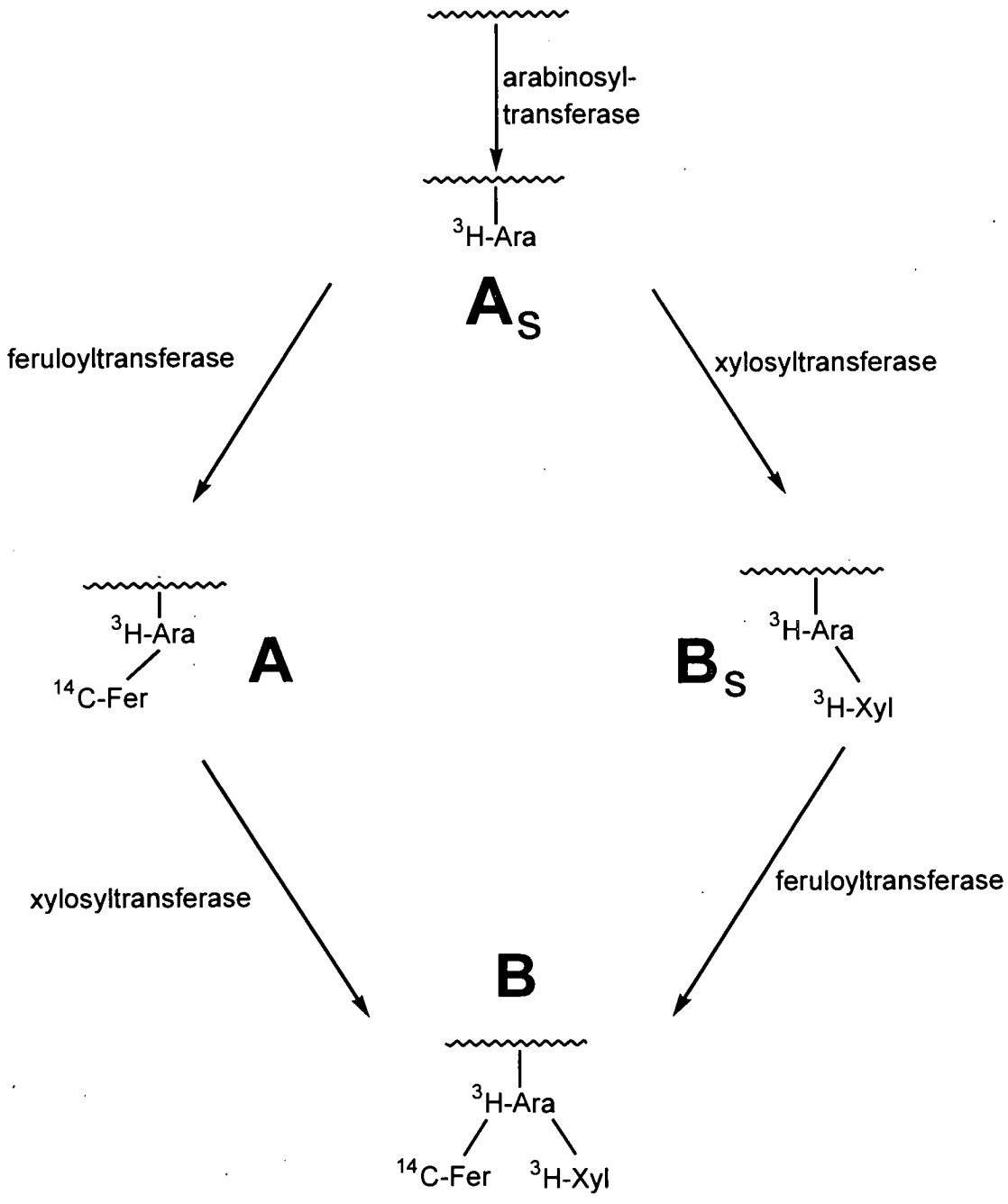


Fig. 4.8: Two hypothetical pathways for the assembly of compound B side chains on the xylans of *Festuca*. Either the feruloylation of arabinose takes place first (to obtain feruloylated arabinose, releasable by mild acid as compound A) and then a xylose residue is added or the reverse is true. ~~~~ = backbone β-(1→4)-xylan chain.

4.10 Degradation of compound B by rat caecal bacteria

Plant cell walls are an important source of nutrients in animal feed. They are utilised for instance by ruminants for the production of milk, meat and wool (Akin, 1993). Before utilisation, the PCW's need to be digested, through the intervention of microbes. However, microbial degradation is subject to restrictions. Three options have been described (Tamminga, 1993) to increase the availability of nutrients from PCW's: an increase of the digestion rate, an increase of the rate of passage and an increase of the capacity of the rumen to hold material. Lowrey *et al.* (1968) have shown that a 12% increase in digestibility resulted in a 30% mean weight gain and approximately 25% less feed per kg of gain in dairy cows.

In view of the unusual structure of compound B (§ 3.5), the question was addressed as to whether this compound is likely to confer resistance to microbial degradation or not. After treatment of a PCW-fragment (compound B) with rat caecal bacteria, degradation products (Fer-Ara, Ara-Xyl, xylose, arabinose and ferulic acid) could be monitored at a very early stage, showing that all enzymes necessary for a rapid breakdown of compound B are present in the rat gut micro-organisms. However, the result might be different with intact feruloylated arabinoxylans of fescue. Dehority (1993) demonstrated that in intact plants, the cellulose digestion for instance decreased as the plant matured.

4.11 General aspects

Recently it has been reported (Myton, 1993) that feruloylated arabinoxylans of cultured *Festuca* cells are relatively resistant to the enzyme mixture Driselase whereas Driselase can act successfully on other graminaceous cell walls (e.g. maize and barley) to release feruloylated oligosaccharides such as Fer-Ara-Xyl (FAX) and Fer-Ara-Xyl_2 (FAXX) [e.g. ~79% of the ferulate was solubilised from maize]. The resistance of *Festuca* feruloyl-arabinoxylans to Driselase means that only a very low percentage of the feruloyl groups were released as FAX and FAXX. The feruloylated material was solubilised by Driselase (~80% as well) but in the form of relatively high molecular weight conjugates. The question of why *Festuca*

feruloyl-arabinoxylan cannot be digested by Driselase to fragments such as FAX and FAXX to any great extent has been investigated in this thesis.

Approximately 80% of the feruloylated material (in both fescue and maize) was solubilised by Driselase. As it could be demonstrated that compound B is not unique to *Festuca arundinacea* (§ 3.12), the unusual (1→2)-linkage within compound B cannot be the (only) cause of the relative Driselase-"resistance".

The structure of compd B is unusual because the disaccharide (compd B_S) containing the (1→2)-linkage is esterified to ferulic acid. Non-feruloylated oligosaccharide with (1→2)-linkages involved have been described elsewhere (Wilkie, 1979). Himmelsbach *et al* (1994) were the first to publish a feruloylated tetrasaccharide (obtained from shoots of *Cynodon dactylon*) with a β-D-Xyl_p-((1→2)-α-L-Ara_f element (compound B connected to a xylobiose group from the xylan backbone). The compound described by Himmelsbach *et al* (1994) would be expected to be hydrolysed to compd B during treatment with dilute TFA under the conditions routinely used in this thesis.

Nevertheless it is suggested that there is a link between the unusual structure of compound B, the feruloylated arabinoxylan of fescue and the relative Driselase-"resistance".

Further work needs to be carried out on this theme to address the missing "link".

Taken together, this thesis has explored some aspects of feruloylated polysaccharides in the primary cell walls of *Festuca arundinacea* with an emphasis on :

* structural analysis
* the process of xylosylation and feruloylation
* microbial degradation of cell wall fragments and
* chemotaxonomy

However, "clearly the plant cell wall is a structure of considerable economic significance, yet much of its biochemistry remains a mystery. A good deal is known about its component molecules, but details of how these are assembled in the growing wall, how they are cemented in place and how wall growth is controlled at the molecular level, are not understood" (after Hadlington, 1991).

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6. Appendix

6.1 Composition of culture media

6.1.1 Composition of cell suspension culture media

6.1.1.1 *Festuca arundinacea* (tall fescue grass)

Murashige and Skoog, basal salts*	4.4 g/l
Glucose	20.0 g/l
pH 5.7	

6.1.1.2 *Rosa sp.*, cv. Paul's Scarlet (rose)

$CaCl_2 \times 2\ H_2O$	74 mg/l
KH_2PO_4	140 mg/l
KCl	750 mg/l
$NaNO_3$	850 mg/l
$MgSO_4 \times 7\ H_2O$	250 mg/l
$MnSO_4 \times 4\ H_2O$	1 mg/l
H_3BO_3	0.2 mg/l
$ZnSO_4 \times 7\ H_2O$	0.5 mg/l
KI	0.1 mg/l
$CuSO_4 \times 5\ H_2O$	0.02 mg/l
$CoCl_2 \times 6\ H_2O$	0.01 mg/l
$Na_2MoO_4 \times 2\ H_2O$	0.02 mg/l
$FeCl_3 \times 6\ H_2O$	5.4 mg/l
$Na_2EDTA \times 2\ H_2O$	7.4 mg/l
2,4-D-2,4-Dichlorophenoxyacetic acid	1.0 mg/l

* Murashige and Skoog, basal salts (no phytohormones or sucrose/glucose) Sigma Chemical Co. (Murashige and Skoog, 1962)

6.1.2 Composition of Scott and Dehority medium

Bacto-casitone (Tryptone)	1.0 g
Yeast extract	0.25 g
Sodium hydrogen carbonate	0.4 g
Mineral I solution	15.0 ml

Mineral II solution	15.0 ml
V.F.A. solution	7.0 ml
Vitamin solution	1.0 ml
Haemin solution	0.1 ml
Menadione (10 mg/100 ml 70% EtOH)	0.5 ml
Water	62.0 ml
Resazurin (0.1%)	1 drop
Cysteine HCl	0.1 g

The medium was boiled before cysteine was added and the pH was adjusted to 7.8 with 1 M NaOH.

Mineral I solution:

K_2HPO_4	3.0 g

The solution was made up to 1 litre with dH_2O.

Mineral II solution:

KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	6.0 g
NaCl	6.0 g
$MgSO_4$	0.6 g
$CaCl_2$ (dry)	0.6 g

The solution was made up to 1 litre with dH_2O.

V.F.A. solution:

Acetic acid	20.0 ml
Iso-butyric acid	1.0 ml
Iso-valeric acid	1.2 ml
n-Valerate	1.2 ml
2 Methyl butyrate	1.2 ml

The V.F.A. solution was made up to 1 litre with dH_2O.

Vitamin solution:

Pyridoxine HCl	0.2 g
Riboflavine	0.2 g
Cobalanine	0.005 g
Ca-d-Pantothenate	0.2 g
Thianine HCl	0.2 g
p-Amino benzoic acid	0.01 g
Biotin	0.005 g
Nicotinamide	0.2 g
Folic acid	0.005

Added 1 M NaOH to dissolve and then made up to 1 litre with dH_2O.

Haemin solution:

0.1 g Heamin was dissolved in 10 ml absolute EtOH and then made up to 100 ml with 0.05 M NaOH.

6.2 McIlvaine buffer

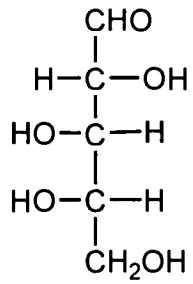
Citric acid - Na_2HPO_4 (McIlvaine) buffer, pH approximately 4.0 - 5.0

x ml 0.2 M citric acid monohydrate and y ml 0.4 M Na_2HPO_4 were mixed to obtain double strength.

pH	x ml 0.2 M citric acid	y ml 0.4 M Na_2HPO_4
4.0	61.45	38.55
4.2	58.60	41.40
4.4	55.90	44.10
4.6	53.25	46.75
4.8	50.70	49.30
5.0	48.50	51.50

Dawson *et al.* (1989)

6.3 Formulae



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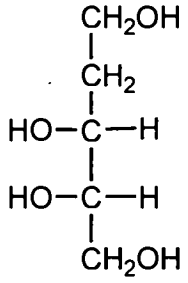
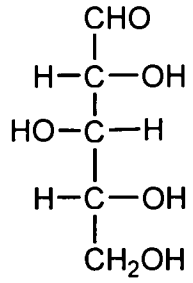
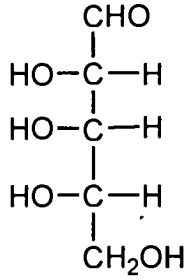


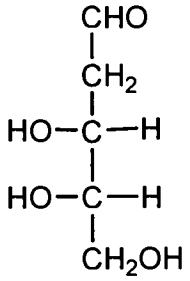
2-deoxy-L-Arabinitol



D-Xylose



L-Ribose



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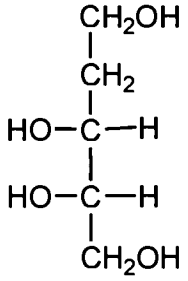
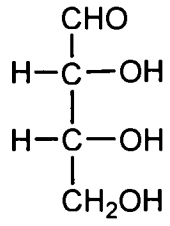
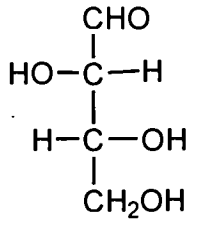


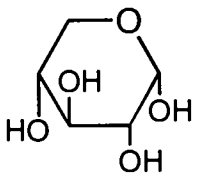
2-deoxy-L-Ribitol



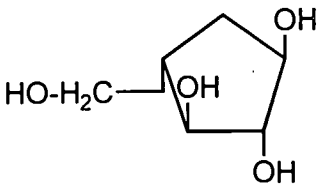
D-Erythrose



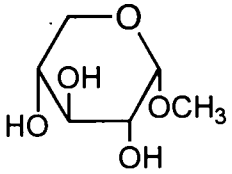
D-Threose



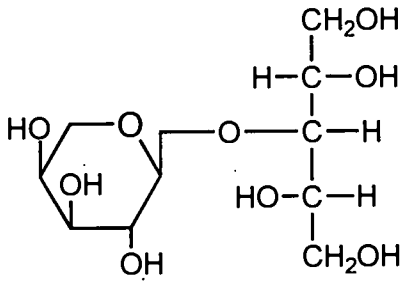
α-D-Xylopyranose



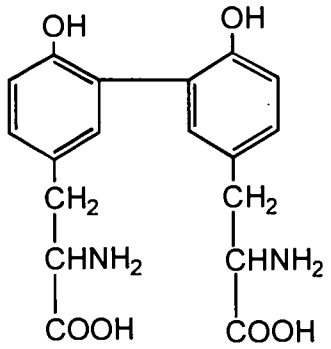
α-L-Arabinofuranose



Methyl α-D-xylopyranoside



Galactosyl-1,3-arabinitol



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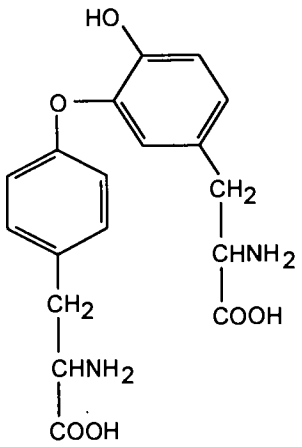
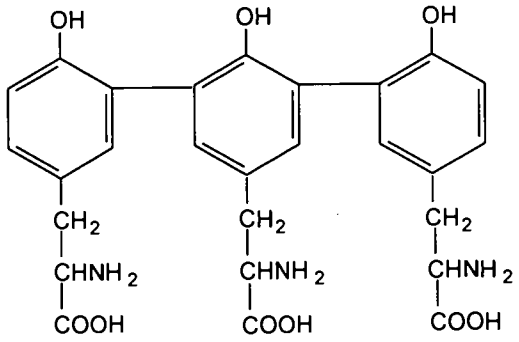
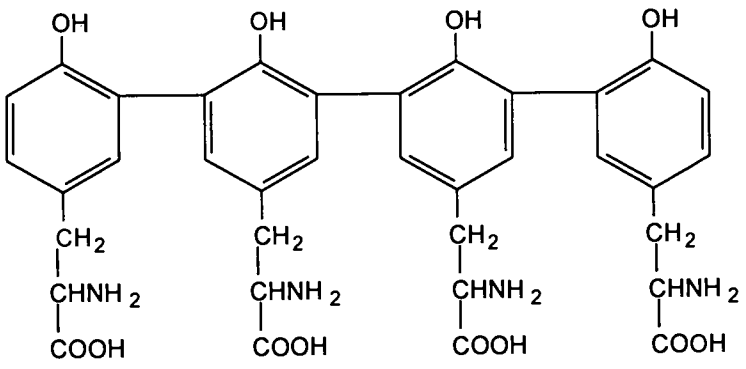


Isodityrosine



Trityrosine



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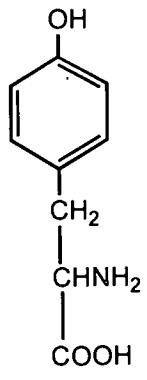

Tyrosine

7. Publications

Paper presented at the 4th Scottish Cell Wall Group Meeting in Glasgow in April 1994

Partial characterisation of a novel feruloyl disaccharide from *Festuca* cellwalls

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Background. Ferulic acid is an important phenolic component in primary cell walls. Consequently it has attracted much attention to investigate the role of this wall component. A correlation has been reported between the release of ferulic acid after alkaline hydrolysis and microbial degradation (1). Kamisaka *et al.* (2) reported a decrease in coleoptile elongation correlated with diferulic acid. Fry (3, 4) proposed a cross-linkage of feruloylated polysaccharides by diferulic acid to restrict cell wall extensibility. Ferulic acid was found to be esterified with specific polysaccharides (5). Recently it has been discovered (6) that feruloylated polysaccharides of an arabinoxylan of *Festuca* are resistant to the enzyme mixture "Driselase", whereas "Driselase" can act successfully on other graminaceous cell walls (e.g. maize) and release feruloylated oligosaccharides such as Fer-Ara-Xyl and Fer-Ara-Xyl_2.

Materials and Methods. Suspension cultured cells of *Festuca arundinaceae* were supplied with L-[1-^{3}H]arabinose, and alcohol-insoluble residues (AIR) were prepared. Feruloylated oligo- and polysaccharides were released from AIR by mild acid hydrolysis and separated by paper chromatography. With the aid of UV-light (366 nm) eight blue fluorescent zones (A to H; H = origin) were detected, strongly suggesting feruloyl derivatives (7). Zone B (designated compound B) was characterized in terms of sugar composition, reducing termini, anomeric configuration and linkage within the molecule.

Results. Alkaline hydrolysis of compound B yielded the de-feruloylated compound (designated B_S). Arabinose and xylose were the only sugars found after severe acid hydrolysis. The ratio between arabinose and xylose was estimated at 1.06 : 1.0. Reduction with sodium borohydride yielded arabinitol and xylose. Sequential $NaBH_4$-, $NaIO_4$-, $NaBH_4$- and TFA-treatment of compound B yielded [^{3}H]glycerol showing a (1→2)-linkage of xylose to arabinose. Upon paper electrophoresis in molybdate buffer at pH 3, the compound migrated alongside 2-deoxyarabinitol indicating a (1→2)-linkage within the molecule. The molecule was found to be hydrolysed by β-xylosidase.

Conclusions. Compound B_S is a disaccharide consisting of arabinose and xylose with arabinose as reducing terminus. The results of the sodium periodate oxidation and of the paper electrophoresis are consistent with O-2 linkage of xylose to arabinose. The designated structure of compound B is 5-O-feruloyl-(2-O-β-D-xylopyranosy)-L-arabinose.

References.(1) Jung & Fahey (1981) J. Agric. Food Chem. 29: 817 - 820; (2) Kamisaka *et al.* (1990) Physiologia Plantarum 78: 1 - 7; (3) Fry (1986) Ann. Rev. Plant Physiol. 37: 165 - 86; (4) Fry (1989) Modern Methods in Plant Analysis, New Series 10: 12 - 36; (5) Fry (1982) Biochem J. 203: 493 - 504; (6) Myton (1993) PhD thesis, Edinburgh University; (7) Kato & Nevins (1985) Carbohydrate Research 137: 139 - 150

Poster presented at the Botanikertagung in Bayreuth (Germany) in September 1995

Abstract Number P 9.17

Unusual structure of *Festuca* feruloyl-arabinoxylan conferring resistance enzymic hydrolysis

Gundolf Wende and Stephen C. Fry

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Background. Ferulic acid is thought to be an important phenolic component in primary cell walls. Feruloylated oigosaccharides of an arabinoxylan of *Festuca* show an unusual resistance to the enzyme mixture Driselase, whereas Driselase can act successfully on other graminaceous cell walls to release feruloylated oligosaccharides.

Material and Methods. Radio-labelled alcohol-insoluble residue (AIR) were prepared from suspension cultured cells of *Festuca arundinacae* (fed with L-[1-^{3}H]arabinose). Feruloylated [^{3}H]oligosaccharides were released from AIR by mild acid hydrolysis. The major compound (compound B) was characterised in terms of sugar composition, reducing termini, anomeric configuration and linkage.

Results. Compound B was resistant to Driselase but was hydrolysed by β-xylosidase, yielding arabinose and xylose as the sole sugars in a ratio 1.06 : 1.00. Reduction with sodium borohydride gave arabinitol and xylose. Sodium periodate oxidation yielded [^{3}H]glycerol. Upon paper electrophoresis in molybdate buffer at pH 3, the compound co-migrated with 2-deoxyarabinitol.

Conclusions.. Compound B is provisionally identified as 5-*O*-(*E*)-feruloyl-2-*O*-β-D-xylopyranosyl-L-arabinose. This novel structure is proposed to account for the resistance of *Festuca* feruloyl-arabinoxylan to enzymic hydrolysis.

Poster presented at the 5th Scottish Cell Wall Group Meeting in Edinburgh in June 1995

Unusual resistance of feruloylated oligosaccharides from *Festuca arundinacea* to fungal attack

Gundolf Wende

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Background. The main phenolics in growing cell walls of grasses are feruloyl (Fer) and *p*-coumaroyl esters of polysaccharides; their roles have not been elucidated completely [1–3)] Myton [4] discovered that feruloylated arabinoxylan of *Festuca* shows an unusual resistance to enzymic digestion (Driselase), whereas Driselase can act successfully on other graminaceous cell walls (e.g. maize and barley) to release feruloylated oligosaccharides e.g. Fer-Ara-Xyl (FAX) and FAXX [5]. Most of the feruloylated material was solubilised from *Festuca* walls by Driselase in the form of high-M_r conjugates; very little was released as feruloylated di- and trisaccharides. The present work aimed to deduce the linkage of feruloyl groups in the *Festuca* cell wall.

Data. Radio-labelled alcohol-insoluble residues (AIR) were prepared from suspension cultured cells of *Festuca arundinacea* that had been fed L-[1-^{3}H]arabinose. The AIR was hydrolysed with 0.1 M TFA, and two major feruloylated [^{3}H]oligosaccharides (B and C) were purified by paper and gel-permeation chromatography. Alkaline hydrolysis of B was a single-step reaction yielding E-ferulate and a disaccharide (B_S), which gave L-arabinose and D-xylose (1.06:1) on hydrolysis in 2 M TFA. Only the arabinose group of B_S was reducible by $NaBH_4$. The resistance of B to 0.1 M TFA thus indicated a Xyl*p* residue. β-Xylosidase digested B to yield D-xylose and 5-*O*-feruloylarabinose, suggesting a β-bond. A linkage to the 2-position of the Ara was shown by $NaIO_4$-oxidation (yielding [^{3}H]glycerol) and confirmed by the immobility of reduced B_S [β-Xyl*p*-(1→2)-arabinitol] on molybdate electrophoresis.

Alkaline hydrolysis of C was a single-step reaction yielding E-ferulate and a trisaccharide (C_S). Acid hydrolysis of C_S gave L-arabinose and D-xylose (~1:2). Hydrolysis of compound C by Driselase released compound B.

Compounds B and C are structurally related to a feruloyl tetrasaccharide that was isolated from *Cynodon dactylon* but only characterised by NMR (6).

Conclusions. Compound B is (5-*O*-*E*-feruloyl)(2-*O*-β-D-xylopyranosyl)-L-arabinose. Compound C is based on a core of B and has one additional xylose residue. These novel structures are proposed to account for the inability of enzymic hydrolysis to yield small feruloylated fragments from *Festuca* feruloyl-arabinoxylan.

References. [1] Yamamoto E *et al.* (1989), ACS Symp Ser 399, Ed N.G.Lewis & M.G.Paice: 68–88. [2] Fry SC, Miller JG (1989) *ibid* 33–46. [3] Hatfield DD *et al.* (1991) *Anal Biochem* 194: 25–33. [4] Myton (1993) PhD thesis, Edinburgh University. [5] Kato Y, Nevins DJ (1985) *Carbohydr Res* 137: 139–150. [6] Himmelsbach DS *et al.* (1994) *Magn Reson Chem* 32: 158–165.

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UNUSUAL FERULOYL-OLIGOSACCHARIDES FROM *FESTUCA* ARABINOXYLAN: THEIR ROLE IN RESISTANCE TO ENZYMIC DIGESTION

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